Mycobacterium xenopi and related organisms isolated from stream waters in Finland and description of Mycobacterium botniense sp. nov.

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Three scotochromogenic *Mycobacterium xenopi*-like organisms were isolated from stream waters in Finland. These strains grew at 36–50 °C but not at 30 °C. One of the three strains was fully compatible with the *M. xenopi* type strain according to GLC-MS, biochemical tests, and 16S rDNA and 16S–23S rDNA internal transcribed spacer (ITS) sequencing. Two of the strains closely resembled *M. xenopi* in lipid analyses and biochemical tests, but analysis by GLC-MS verified the presence of two new marker fatty acids (2,4,6,x-tetramethyl-eicosanoic acid and 2,4,6,x,x-pentamethyl-docosanoic acid). The 16S rDNA and ITS region sequences of these two strains differed from those of *M. xenopi* and other previously described mycobacterial sequences. Therefore, the strains are regarded as new species of slow-growing mycobacteria, for which the name *Mycobacterium botniense* sp. nov. is proposed. The chemical, physical and microbiological quality of the water reservoirs of *M. xenopi* and *M. botniense* are described. As far as is known, this is the first time that *M. xenopi* has been isolated from natural waters. The strains of *M. botniense* sp. nov. (E347[T] and E43) have been deposited in the ATCC as strains 700701[T] and 700702, respectively.

INTRODUCTION

In a project established to evaluate natural reservoirs of environmental mycobacteria, a series of water samples from Finnish streams was examined (Iivanainen *et al.*, 1993). Mycobacterial isolates were analysed for the whole-cell lipid composition by GLC and classified according to their GLC profiles and appropriate biochemical and growth characteristics. The present study is a polyphasic taxonomic characterization of *Mycobacterium xenopi* and related mycobacterial strains isolated from these natural waters. Among the 757 isolates analysed, three strains were tentatively identified as *M. xenopi*. Further analyses

indicated that two of the three isolates formed a clear cluster, distinct from *M. xenopi sensu stricto*. It is proposed that they represent a previously unidentified species, for which the name *Mycobacterium botniense* sp. nov. is suggested.

METHODS

Bacterial strains. *M. xenopi* strains present in the strain collection of the Department of Clinical Microbiology, Kuopio University Hospital, Finland were reanalysed for lipid profiles, in order to evaluate the stability of fatty acid and mycolic acid cleavage product (MACP) profiles among *M. xenopi*. These strains included *M. xenopi* ATCC 19250[T] and ATCC 19971, 25 clinical isolates (27, 69, M116, 150, 1244, 1411, La 2593, 9533, 13082, 17598, 21074, 22145, 22259, 30476, 31631, 31686, 32479, 32746, 36267, 36342, 41334, 45979, 55507, 55508, 59389) and one isolate from a pig (4264). Three environmental mycobacterial strains (E43, E341 and E347[T]) were included in the study. They originated

from two separate brook waters examined as described previously in detail (Iivanainen *et al.*, 1993). The strains were stored in Middlebrook 7H9 broth (Difco) at −80 °C.

Characterization of strains. In the present study, all isolates were tested for growth rate, pigment production, and growth at 20, 30, 37, 42 and 45 °C (Lévy-Frébault & Portaels, 1992). Growth of the environmental strains was also tested at 50 °C.

Lipid analyses. For chromatographic analyses, the strains were cultured on Middlebrook 7H11 agar supplemented with Middlebrook OADC enrichment (Difco) at 42 °C for 6–8 weeks. Fatty acid methyl esters and alcohols were prepared by acid methanolysis, and analysed and identified as reported previously (Torkko *et al.*, 1998).

Two environmental strains (E43 and E347$^{\mathrm{T}}$) were also analysed for cell wall mycolic acid composition by HPLC as previously described (Butler *et al.*, 1992).

Biochemical tests. The strains were tested for urease, aryl-sulfatase (3-d and 10-d), pyrazinamidase, nitrate reduction and Tween 80 hydrolysis as described previously (Torkko *et al.*, 1998). Furthermore, the following properties of the environmental strains were determined: growth in the presence of 5% NaCl, acid phosphatase, semi-quantitative catalase (>45 mm foam) and heat-stable (68 °C) catalase (Lévy-Frébault & Portaels, 1992). *β*-Galactosidase was determined by using commercial discs as recommended by the manufacturer (Rosco).

16S rDNA sequencing and phylogenetic analysis. Amplification of the partial 16S rRNA gene and sequencing of both strands of the amplified DNA fragments were performed on selected strains as described previously (Koukila-Kähkölä *et al.*, 1995). All three environmental isolates were sequenced for the complete 16S rDNA. A BLAST search (EBI) was done with 16S rDNA sequences and the closest hits were chosen for further analysis. Sequences of the investigated strains were aligned with selected published sequences using the PILEUP program in the GCG software package (Devereux *et al.*, 1984). A phylogenetic tree was constructed using the software package TREECON (Van de Peer & De Wachter, 1994). Similarity values were calculated by the Kimura (1980) method and the neighbour-joining method was used to construct the dendrogram (Saitou & Nei, 1987).

Internal transcribed spacer region sequencing. The internal transcribed spacer (ITS) region between 16S and 23S rDNAs was amplified by using primer 5′-GTACACACCGCCCG-TCA-3′ in combination with 5′-TCTCGATGCCAAGGC-ATCC-3′ following the method described by Roth *et al.* (1998). PCR was performed in a 50 µl reaction mixture containing 200 µM dNTP, 1× Dynazyme buffer, 25 pmol each primer and 1 U Dynazyme polymerase (Finnzymes). The thermal cycler was programmed as described previously for 16S rDNA (Koukila-Kähkölä *et al.*, 1995). The amplification products were purified with MicroSpin S-400 HR columns (Pharmacia) and sequenced directly with the same primers used in amplification. Sequencing was done by using a Dye Terminator Cycle Sequencing kit and an automated ABI 377 DNA Sequencer (Perkin Elmer). The Staden software package was used for sequence assembly and for comparing the ITS sequences between *M. botniense* sp. nov. and *M. xenopi* (Bonfield *et al.*, 1995).

Nucleotide sequence accession numbers. The 16S rDNA sequence accession numbers of the mycobacterial strains used as references in the phylogenetic analyses are as follows:

M. xenopi, X52929; *Mycobacterium triviale*, X88924; *Mycobacterium nonchromogenicum*, X52928; *Mycobacterium terrae*, X52925; *Mycobacterium branderi*, X82234; *Mycobacterium celatum*, L08170; *M. celatum* type 3, Z46664; *Mycobacterium shimoidei*, X82459; *Mycobacterium conspicuum*, X88922; *Mycobacterium leprae*, X55022; *Mycobacterium tuberculosis*, X58890; *Mycobacterium malmoense*, X52930; *Mycobacterium bohemicum*, U84502; *Mycobacterium intracellulare*, X52927; *Mycobacterium paratuberculosis*, X53934; *Mycobacterium avium*, X52918; *Mycobacterium gordonae*, X52923; *Mycobacterium heidelbergense*, AJ000684; *Mycobacterium simiae*, X52931; *Mycobacterium triplex*, U57632; and *Nocardia asteroides*, Z36934.

Environmental analyses. Characteristics of the drainage areas, and chemical, physical and microbiological quality of the water reservoirs in which *M. botniense* sp. nov. and *M. xenopi* were found were analysed as described previously in detail (Iivanainen *et al.*, 1993).

RESULTS

Lipid analyses

The fatty acid patterns of all 26 *M. xenopi* strains isolated from clinical or veterinary sources and the ATCC strains consisted of saturated and unsaturated straight-chain fatty acids as well as 10-methyl-octa-decanoic acid (tuberculostearic acid). The known prominent alcohol markers of *M. xenopi*, 2-docosanol and 2-eicosanol (Alugupalli & Larsson, 1992), were detected, in addition to hexacosanoic acid (26:0) (Table 1). Dodecanoic acid (12:0) was also found to be a significant marker present in all strains (except strain 41334), in which it was present at a mean relative amount of 7·7%. The three environmental isolates also had GLC fatty acid and alcohol profiles very similar to that of *M. xenopi*. However, two of them (E43 and E347$^{\mathrm{T}}$), although otherwise similar, lacked dodecanoic acid.

GLC-MS analysis of the environmental strains E43 and E347$^{\mathrm{T}}$ highlighted two further differences. Firstly, an additional compound was found to co-elute with the peak of 2-docosanol. Secondly, 2-tetracosanol, a minor marker of *M. xenopi* (Alugupalli & Larsson, 1992), was replaced by another compound which had an approximately similar retention time. These two compounds were found to be additional fatty acid methyl esters. They eluted between 20:0 and 22:0, and between 22:0 and 24:0, indicating that the main carbon skeletons of the compounds contained 20 and 22 carbons, respectively. The first of the peaks contained 24 carbons and the latter 27 carbons, resulting in mass peaks at m/z 382 and 424, which also indicates the presence of four and five methyl branches, respectively. The compounds contained no hydroxyl groups on the basis of trimethylsilyl derivatization (Torkko *et al.*, 1998), nor did they have any double bonds. The mass spectra of the compounds displayed ions at m/z 88 [CH$_3$O-C(OH) = CH(CH$_3$)]$^+$, m/z 101 [CH$_3$O-CO-CH(CH$_3$)-CH$_2$]$^+$ (base peak), m/z 129 and m/z 171, which confirmed the locations of three methyl groups at C-2, C-4 and C-6. The positions of the other

Table 1. Fatty acid and fatty alcohol markers (mean and range of total peak area), useful for the classification of *M. xenopi* and *M. botniense* sp. nov.

Fatty acid and alcohol markers: 1, dodecanoic acid; 2, 2-eicosanol; 3, 2,4,6,x-tetramethyl-eicosanoic acid; 4, 2-docosanol; 5, 2,4,6,x,x-pentamethyl-docosanoic acid; 6, tetracosanoic acid; 7, hexacosanoic acid.

Strain	Marker						
	1	2	3	4	5	6	7
M. xenopi ATCC strains (n = 2)	6·0 (3·9–8·1)	1·6 (1·2–2·0)	0	11·2 (10·1–12·2)	0	2·2 (1·9–2·2)	10·7 (8·2–13·2)
M. xenopi clinical strains (n = 25)*	7·6 (0·0–12·1)	2·0 (1·6–3·9)	0	11·6 (8·6–13·4)	0	1·8 (1·0–3·9)	11·2 (7·2–21·9)
M. xenopi porcine isolate (n = 1)	7·0	1·6	0	9·9	0	1·1	11·7
M. xenopi environmental isolate E341	7·9	1·8	0	10·9	0	1·7	9·8
M. botniense environmental isolates E43, E347[T]	0	3·1 (2·9–3·3)	16·1 (15·1–17·0)†	3·2 (3·1–3·2)†	1·8 (1·7–1·8)	1·5 (1·1–1·9)	7·0 (5·9–8·1)

* One strain totally lacks dodecanoic acid.

† Amounts estimated from GLC-MS. The peak of 2,4,6,x-tetramethyl-eicosanoic acid has approximately the same retention time as 2-docosanol in GLC.
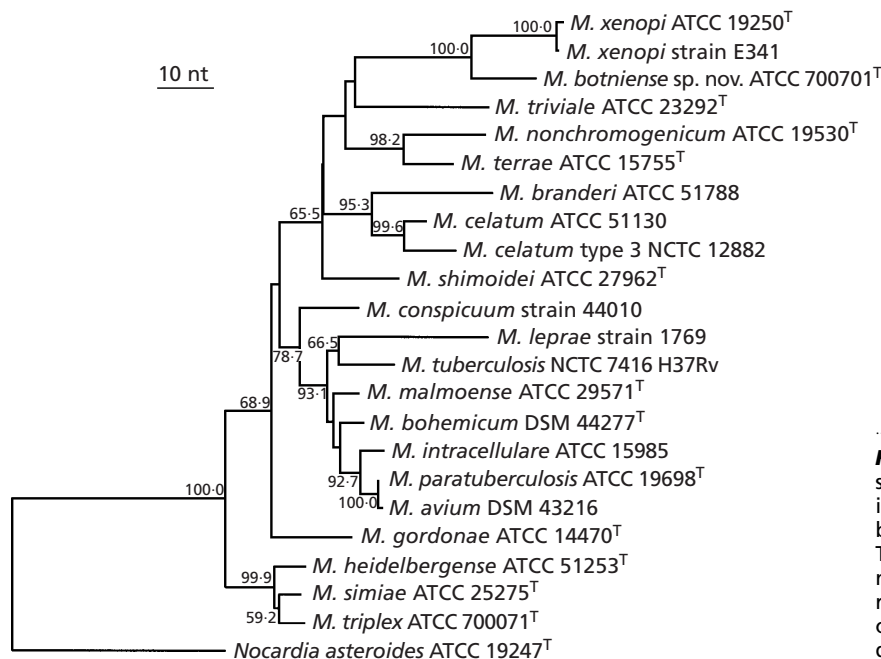

Fig. 1. Phylogenetic tree based on 16S rDNA sequence showing the relationships of the investigated strains and selected species belonging to the genus *Mycobacterium*. This tree was constructed by using the neighbour-joining method. The tree was rooted by using *Nocardia asteroides* as the outgroup. The bar indicates a 10 nucleotide difference.

methyl branches were not determined. Accordingly, the first of the compounds was identified as 2,4,6,x-tetramethyl-eicosanoic acid (2,4,6,x-tetramethyl-20:0) and the latter as 2,4,6,x,x-pentamethyl-docosanoic acid (2,4,6,x,x-pentamethyl-22:0).

The HPLC mycolic acid profiles of the strains E43 and E347[T] were very similar to the known profile of *M. xenopi* (Tortoli & Bartoloni, 1996). They shared the same peaks, although the peaks had slightly different relative heights.

16S rDNA sequencing and phylogenetic analysis

The complete 16S rDNA of the three environmental strains (E43, E341 and E347[T]) was sequenced. In addition, the 16S rDNA of two randomly selected clinical *M. xenopi* isolates (9533 and 45979), the clinical *M. xenopi* isolate lacking dodecanoic acid (41334), and the porcine isolate (4264) was partially sequenced. The partial sequence covered the region corresponding to *Escherichia coli* positions 30–900, which includes two of the most hypervariable regions. All of these 16S rDNA sequences were compared with that of the *M. xenopi* type strain ATCC 19250[T] (Rogall *et al.*, 1990). Compared with the *M. xenopi* type strain, single nucleotide differences were detected in the variable regions of the 16S rDNA in porcine and clinical strains, and in one environmental isolate (E341). Strain 41334 had a difference of two nucleotides when compared with the type strain. The other two environmental isolates (E43 and E347[T]) were identical. Their 16S rDNA sequence differed from all sequences

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M. xenopi ATCC 19250ᵀ   AGGGAGCACC GTAAACGCAT CCCG------ CGTGGGGT-- -GTGGGTTCG G---------  42
M. xenopi (Mxe-A)       .......... .......... ....------ .........-- -......... .---------  42
M. xenopi (Mxe-B)       .......... .......... ....TGTTG- -........-- -......... .---------  46
M. xenopi E341          .......... .......... ....CGTGGG -........-- -......... .---------  47
M. xenopi (Mxe-C)       .......... .......... ....CGTGGG -........-- -......... .---------  47
M. botniense sp. nov. E347ᵀ  .......... -.CGTGTTT. ....GGTTCT ....T..AG C.G....... .GGGAGCGTC  59


M. xenopi ATCC 19250ᵀ   ---------- ---------- ----CGTGTT GTGGCGTCGG GCCGAGGTGT TGGGCAGCAG  78
M. xenopi (Mxe-A)       ---------- ---------- ----...... .......... .......... ..........  78
M. xenopi (Mxe-B)       ---------- ---------- ----...... .......... .......... ..........  82
M. xenopi E341          ---------- ---------- ----...... .......... .......... ..........  83
M. xenopi (Mxe-C)       ---------- ---------- ----...... .......... .......... ..........  83
M. botniense sp. nov. E347ᵀ  AATATCAATG TTGACGGGGC CGGC.CC..G ....G..T.. TTG.GT.G.G ..C....... 119


M. xenopi ATCC 19250ᵀ   GCAGTAACCG CCGGCACACT GTTGGGTTTT GAGGCAACAC CCGTGGTGGT GTTGTGCT-- 136
M. xenopi (Mxe-A)       .......... .......... .......... .......... .......... .........-- 136
M. xenopi (Mxe-B)       .......... .......... .......... .......... .......... .........-- 140
M. xenopi E341          .......... .......... .......... .......... .......... .........-- 141
M. xenopi (Mxe-C)       .......... .......... .......... .......... .......... .........-- 141
M. botniense sp. nov. E347ᵀ  --.TG.G... ..A....... .......... .......... .-.GTT.... ......GCCT 176


M. xenopi ATCC 19250ᵀ   CCGCGTGGTG GCGGGGTGTG GTGTTTGAGT GTTGGATAGT GGTTGCGAGC ATCTGGCAAA 196
M. xenopi (Mxe-A)       .......... .......... .......... .......... .......... .......... 196
M. xenopi (Mxe-B)       .......... .......... .......... .......... .......... .......... 200
M. xenopi E341          .......... .......... .......... .......... .......... .......... 201
M. xenopi (Mxe-C)       .......... .......... .......... .......... .......... .......... 201
M. botniense sp. nov. E347ᵀ  .AC....... .T........ .......... CG........ .......... ...GA-.... 235


M. xenopi ATCC 19250ᵀ   GACTGTGGTA AGCGGTTTTT GTTGAGTG-T TTTCTGGTGT 235
M. xenopi (Mxe-A)       .......... .......... ........-. .......... 235
M. xenopi (Mxe-B)       .......... .......... ........-. .......... 239
M. xenopi E341          .......... .......... ........-. .......... 240
M. xenopi (Mxe-C)       .......... .......... ........-. .......... 240
M. botniense sp. nov. E347ᵀ  A.GC....C. -...T..... ..GTCT..G. ...G..-... 273
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Fig. 2. Alignment of 16S–23S rDNA ITS sequences including the investigated strains and *M. xenopi* sequevar sequences. The sequence of *M. xenopi* was used as the reference sequence. The length of the ITS is indicated at the end of the sequences in nucleotides. Dots indicate identity and hyphens represent alignment gaps. Accession numbers of the *M. xenopi* sequevars are as follows (Roth *et al.*, 1998): Mxe-A, Y14190; Mxe-B, Y14191; and Mxe-C, Y14192.

available in the EMBL database. A similarity search indicated that this sequence was closest to that of the *M. xenopi* type strain, although there were 22 nucleotide differences detectable. A phylogenetic tree was constructed to show the location of the investigated isolate among slow-growing mycobacteria (Fig. 1).

ITS sequencing of environmental isolates

According to the ITS sequence analysis, E341 was identical to *M. xenopi* sequevar C (Fig. 2) (Roth *et al.*, 1998). The ITS sequences of strains E43 and E347ᵀ were identical, but unique. They showed the closest similarity to *M. xenopi*, despite the fact that there was a more than 30 nucleotide difference in size and only 80 % similarity to any known *M. xenopi* sequevar (Fig. 2). The genetic evidence revealed by 16S rDNA and ITS region sequencing indicates that these two isolates belong to a previously unclassified species.

Growth temperature ranges and biochemical test results

All three environmental strains, E43, E341 and E347ᵀ, were originally isolated at 42 °C. In subculture, they grew at 36–50 °C, but not at 30 °C; this was also the case for the *M. xenopi* ATCC strains, as well as the clinical and veterinary *M. xenopi* strains. At 50 °C, strain E341 grew well, whereas growth of E347ᵀ and E43 was dysgonic (Table 2). Strains E43 and E347ᵀ preferred egg media to Middlebrook. Subculture on Middlebrook 7H11 agar yielded barely visible colonies after 6 weeks incubation. Both E43 and E347ᵀ grew best on glycerol egg medium, pH 6·5. Strains E43 and E347ᵀ were initially isolated on glycerol egg medium (pH 5·5) and on pyruvate egg medium (pH 5·5), respectively, even though these media were poor supporters of growth. All strains were positive for 10-d arylsulfatase and pyrazinamidase, and negative for urease, nitrate reduction and Tween 80 hydrolysis. In the 3-d arylsulfatase test, strains E43 and E347ᵀ were negative, in contrast to the positive results obtained with all *M. xenopi* strains examined in this study. Strain E341 was positive for heat-stable catalase, in contrast to strains E43 and E347 which were negative. All of the environmental strains were negative for semi-quantitative catalase, β-galactosidase, acid phosphatase and 5 % NaCl tolerance.

Environmental data

The sampling site of the environmental, genuine *M. xenopi* isolate (E341) was located approximately 100 km east from the coast of the Gulf of Bothnia in a rural region without any adjacent population, meaning that contamination by domestic animals or humans is unlikely. The area is rich in peatland. This was reflected by the quality of the stream water which was slightly acidic (pH 5·4) and had a high content of organic matter, as indicated by the colour value (360 mg Pt l⁻¹) and chemical oxygen demand (94 mg KMnO₄ l⁻¹). The total viable count of mycobacteria was 730 c.f.u. l⁻¹.

Strains E43 and E347ᵀ were isolated from the same geographic district as strain E341; the distance between these two sampling sites was 40 km. Strains E43 and E347ᵀ were isolated from the same stream water sample, but by different decontamination methods (Iivanainen *et al.*, 1993). In this stream water, the total viable count of mycobacteria was high (1200 c.f.u. l⁻¹). The drainage area of the stream contained 40 % peatland. The content of organic matter in the water was high (colour, 280 mg Pt l⁻¹; chemical oxygen demand, 84 mg KMnO₄ l⁻¹), and the water was acidic (pH 4·7) and exhibited a very poor buffering capacity (alkalinity, 0·09 mmol HCO₃⁻ l⁻¹).

Table 2. Growth and biochemical characteristics of environmental *M. xenopi* and *M. xenopi*-like strains assigned as *M. botniense* sp. nov.

Test	*M. xenopi* E341	*M. botniense*	
		E347$^{\mathrm{T}}$	E43
Growth at 42 °C on:			
Middlebrook 7H11	+	−*	−*
Glycerol egg, pH 6·5	+	+	+
Glycerol egg, pH 5·5	+	+	+
Pyruvate egg, pH 6·5	+	−	−
Pyruvate egg, pH 5·5	+	+	+
Middlebrook 7H11 supplemented with haemin	+	−*	−*
Growth at 30 °C	−	−	−
Growth at 50 °C	+	+†	+
3-d arylsulfatase	+	−	−
10-d arylsulfatase	+	+	+
Pyrazinamidase	+	+	+
Catalase (>45 mm foam; semi-quantitative)	−	−	−
Catalase (heat-stable)	+	−	−
β-Galactosidase	−	−	−
Acid phosphatase	−	−	−
Urease	−	−	−
Nitrate reduction	−	−	−
Tween 80 hydrolysis	−	−	−

* Growth only observed from high inocula as dysgonic colonies.

† Growth visible after 8 weeks incubation.

DISCUSSION

The results of 16S rDNA and ITS region sequencing, combined with those of phenotypic analyses, showed that the three environmental strains examined could be divided into two entities, one differing from earlier known species (strains E43 and E347$^{\mathrm{T}}$) and the other identical to *M. xenopi sensu stricto*. Thus, it is proposed that strains E43 and E347$^{\mathrm{T}}$ are assigned to a new species, *M. botniense* sp. nov.

Strains E43 and E347$^{\mathrm{T}}$ closely resembled *M. xenopi* in lipid analyses by GLC and HPLC, and biochemical testing. However, 16S rDNA and ITS sequence analyses of these strains highlighted differences which distinguished them from all other mycobacteria previously described. Moreover, a detailed GLC-MS analysis revealed the presence of two new marker fatty acids (2,4,6,x-tetramethyl-20:0 and 2,4,6,x,x-pentamethyl-22:0) in the profiles of both strains. As far as we are aware, *M. botniense* sp. nov. has not been isolated from patients. This implies that the proposed species is saprophytic, or that it has been misidentified as *M. xenopi*. Since the growth characteristics, biochemical properties and fatty acid profiles of *M. xenopi* and *M. botniense* are very similar, their separation may be difficult in a routine diagnostic laboratory. However, 16S rDNA sequencing and GLC-MS can differentiate the two species. Characteristics of *M. botniense* that were useful for distinguishing it from *M.*

xenopi included dysgonic growth, especially on Middlebrook 7H11 agar, and a combination of negative results in the heat-stable catalase test and negative results in the 3-d arylsulfatase test.

HPLC analysis showed that both *M. xenopi* and *M. botniense* present two clusters of peaks characterized by their almost overlapping retention times. In genuine *M. xenopi*, the peaks of the first cluster are consistently higher (approximately double) than those of the second. In *M. botniense*, the peak heights in the two clusters are about equal.

In addition to differences in relative amounts of fatty acid and alcohol markers, minor variations may occur in the fatty acid composition within a particular mycobacterial species. As can be seen from the previously published GLC profiles (Butler *et al.*, 1993; Chou *et al.*, 1996; Jantzen *et al.*, 1989; Larsson *et al.*, 1989; Luquin *et al.*, 1989), dodecanoic acid (12:0) may or may not be detected in *M. xenopi*. Among our clinical isolates, only one of the 25 strains analysed, *M. xenopi* strain 41334, did not contain dodecanoic acid.

M. xenopi is a potentially pathogenic mycobacterium which is frequently detected in clinical samples in some parts of the world but, in some geographic areas, it is most uncommon (Elhelou *et al.*, 1997; Falkinham, 1996; Wolinsky, 1992; Yates *et al.*, 1997). In Finland, the recovery of *M. xenopi* from clinical samples is rare

(less than 0·5% among annual isolates of mycobacteria other than tuberculosis cases) (National Public Health Institute, 1998). *M. xenopi* is also uncommon as a veterinary pathogen. In addition to its initial isolation from a laboratory toad by Schwabacher (1959), it has been isolated from a cat (Tomasovic *et al.*, 1976) and, as we have also observed, from swine (Corner *et al.*, 1981; Jarnagin *et al.*, 1971; Thoen *et al.*, 1975). In experimental studies, it has been found to be pathogenic for rabbits (Demoulin-Brahy, 1978).

All mycobacteria, except for *M. tuberculosis*, appear to have reservoirs in the environment (Falkinham, 1996; Portaels, 1995). The same has been believed to apply to *M. xenopi*. To our present knowledge, however, this is the first report of isolation of *M. xenopi* from natural environments, although it has been isolated from man-made environments, e.g. tap waters, especially hot water systems (Bullin *et al.*, 1970; Costrini *et al.*, 1981; Kaustová *et al.*, 1993; McSwiggan & Collins, 1974; Sniadack *et al.*, 1993; Wright *et al.*, 1985), and sewage (Szabó *et al.*, 1982). The sampling site of the environmental *M. xenopi* isolate was not downstream of any recognizable source of human or animal contamination. The same water also harboured isolates of the *M. avium* complex and *M. malmoense* (Iivanainen *et al.*, 1994; Katila *et al.*, 1995). Peatlands and high organic matter content are apparently important to many potentially pathogenic mycobacteria in the natural environment (Iivanainen *et al.*, 1993, 1999). In addition to this novel mycobacterial species, *M. botniense* sp. nov., several other unidentified mycobacterial groups have been isolated from stream waters, but their taxonomic status and pathogenicity are still unclear (P. Torkko, E. Iivanainen & M.-L. Katila, unpublished results).

Separation of *M. botniense* from other slow-growing pigmented mycobacteria

If conventional identification systems utilizing only biochemical tests are used for identification, it could be possible to misidentify *M. botniense* sp. nov. as a strain of the yellow-pigmented *M. avium* complex or *M. celatum*. GLC can be used to differentiate *M. botniense* from the other pigmented slow-growing mycobacteria, including the *M. avium* complex, but *M. botniense* is easily mistaken for *M. xenopi*. One differentiating characteristic is the lack of dodecanoic acid. Negative results in 3-d arylsulfatase and heat-stable catalase tests are also useful as confirmatory tests. If GLC-MS analysis is carried out, methyl-branched MACPs (2,4,6,x-tetramethyl-20:0 and 2,4,6,x,x-pentamethyl-22:0) can be used marker substances. The most reliable identification is achieved by 16S rDNA sequencing. Even though 2-docosanol has been regarded as a specific marker for *M. xenopi* and *M. shimoidei* (Brander *et al.*, 1992), and now also for *M. botniense*, studies have indicated that minor amounts of 2-docosanol can be detected in *M. celatum* (Butler *et al.*, 1993). The GLC profile of *M. celatum* resembles that of *M. xenopi* and *M. botniense*. In distinguishing *M. xenopi* and *M. botniense* from *M. celatum*, the ratio of 2-docosanol to 2-eicosanol in *M. xenopi*, or the ratio of 2-docosanol plus 2,4,6,x-tetramethyl-20:0 to 2-eicosanol in *M. botniense*, should be greater than one.

Description of *Mycobacterium botniense* sp. nov.

Mycobacterium botniense (bot.ni.en.se. L. gen. *botniense* of Botnia, referring to the Latin name of the province of Finland from which the isolation was made).

Colonies on egg media and on Middlebrook 7H11 agar are small, dysgonic and scotochromogenic, and produce yellow pigment. Visible growth from diluted inocula requires 5–8 weeks. Growth occurs at 37–50 °C. The type strain is positive for 10-d arylsulfatase and pyrazinamidase, and negative for 3-d arylsulfatase, urease, nitrate reductase, semi-quantitative catalase, heat-stable catalase, acid phosphatase, β-galactosidase and 5% NaCl tolerance. Tween 80 is not hydrolysed in 10 d. In GLC-MS analysis, methyl-branched MACPs (2,4,6,x-tetramethyl-20:0 and 2,4,6,x,x-pentamethyl-22:0) are unique markers of *M. botniense*. A phylogenetic tree based on the evaluation of 16S rDNA sequences places *M. botniense* sp. nov. among the slow-growing mycobacteria, close to *M. xenopi*. The strains have been submitted to the American Type Culture Collection as ATCC 700701[T] (= E347[T]) and ATCC 700702 (= E43).

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