Mycobacterium saskatchewanense sp. nov., a novel slowly growing scotochromogenic species from human clinical isolates related to *Mycobacterium interjectum* and Accuprobe- positive for *Mycobacterium avium* complex

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A pigmented, slowly growing *Mycobacterium avium* complex AccuProbe-positive organism was isolated from the sputum and pleural fluid of a 72-year-old female with bronchiectasis. The unusual morphology of the organism prompted further identification by 16S rRNA gene sequencing, revealing a perfect identity with previously uncharacterized strain *Mycobacterium* sp. MCRO 8 (GenBank accession no. X93034), with the closest established species by 16S rDNA analysis being *Mycobacterium interjectum*. HPLC of the organism corresponded to previously obtained patterns identified as *M. interjectum*-like and, upon sequence evaluation of a selection of strains with a similar profile, more were subsequently identified as MCRO 8. A total of 16 strains isolated from human respiratory samples were evaluated in the characterization of this novel species, for which the name *Mycobacterium saskatchewanense* sp. nov. is proposed. The type strain is strain 00-250^T (=ATCC BAA-544^T=DSM 44616^T=CIP 108114^T).

INTRODUCTION

Organisms of the *Mycobacterium avium* complex (MAC) are the non-tuberculous mycobacteria (NTM) species isolated most often in clinical laboratories (Wallace *et al.*, 1997). MAC organisms are often the cause of chronic pulmonary disease in older individuals, lymphadenitis in children and disseminated disease in immunocompromised patients (Wallace *et al.*, 1997) and their detection and

identification is crucial to the administration of appropriate antimicrobial therapy. The availability of a commercial nucleic acid probe (AccuProbe; Gen-Probe) for the identification of MAC species has made the speciation process rapid, accurate and easy, particularly in comparison with the tedious and often ambiguous biochemical tests algorithms.

Strains that are not *M. avium* or *Mycobacterium intracellulare* but that are MAC DNA probe positive, often referred to as MAIX isolates or 'X' strains, have been described previously (Cregan *et al.*, 1992; Smole *et al.*, 2002; Soini *et al.*, 1994, 1996; Tortoli *et al.*, 1997; Viljanen *et al.*, 1993). Sequence-based evaluations of the 32 kDa protein (Soini *et al.*, 1994, 1996), internal transcribed spacer (ITS1) region (Frothingham & Wilson, 1993) and *hsp65* gene (Smole *et al.*, 2002) have suggested that some MAIX strains represent novel species within the complex. We present a clinically significant case of NTM pulmonary infection caused by a novel species of mycobacteria that is MAC AccuProbe positive, but presents with a unique combination of

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Abbreviations: AFB, acid-fast bacillus; ITS, internal transcribed spacer; MAC, *M. avium* complex; NTM, non-tuberculous mycobacteria; PRA, PCR-restriction enzyme analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, ITS1 and partial *hsp65* gene sequences of strains 00-250^T and MB54784 are AY208856–AY208859.

Details of the strains analysed in this study, photos of colonies and an ITS1 alignment are available as supplementary material in IJSEM Online.

genotypic and phenotypic characteristics. Additional strains with identical characteristics were identified from other specimens and have been included in this study.

METHODS

Case history. The patient is a 72-year-old female retired nurse with a 52-year history of recurrent chest infections. She had been on intermittent antibiotic therapy since her late teens but was never hospitalized until 1990, when she developed a right middle lobe pneumonia with lobar collapse. Persistent infection led to a right middle lobectomy in 1991. Pre-operatively, a computed tomography (CT) scan showed extensive bronchiectasis in the right middle lobe, left lower lobe and lingula. Her sputum was negative for acid-fast bacilli (AFB) at that time and her immunoglobulin and sweat chlorides levels were normal. No obvious associations were made for her bronchiectasis, aside from previous recurrent infections. In 1995, the patient's bronchiectasis showed gradual radiographic progression, though cultures for AFB remained negative. New onset of haemoptysis prompted extensive re-evaluation in July 1999. Progressive bronchiectasis was documented and three sputa specimens were positive for AFB identified as *Mycobacterium gordonae* by conventional laboratory methodology. No treatment was offered for this commensal organism, although courses of antibiotics were directed against conventional bacterial pathogens and her haemoptysis settled.

Re-evaluation by a respiratory specialist, including chest X-ray and pulmonary function tests, in August 2000 revealed new left upper lobe cavity lesions. Multiple sputum specimens were positive for AFB (4+), identified by nucleic acid probe as MAC. The patient deteriorated rapidly and was admitted to hospital presenting with a new left pleural effusion and left lower lobe pneumonia. She did not respond to 5 days of intravenous piperacillin/tazobactam and was then placed on a three-drug regimen of ciprofloxacin, azithromycin and amikacin, to which she responded clinically within 48 h. After 5 weeks on this regimen, clinical response was marked and her pleural effusion had resolved, although her sputum samples continued to reveal AFB (1+) with positive culture for *M. avium* based on nucleic acid probes. After 8 weeks of therapy, rifabutin was substituted for amikacin. Compliance with this new regimen was approximately 60%, due to gastrointestinal intolerance, and rifabutin was discontinued after 4 weeks due to a fourfold rise in her hepatic transaminases. After 5 months on the two-drug regimen (ciprofloxacin, azithromycin), sputum was negative for AFB by stain and culture and she was clinically well with stable X-rays. After a treatment ration of 9 months, sputum cultures remain negative for AFB.

Concurrently, one of the strains isolated in summer 2000, strain 00-250^T, was submitted to the National Reference Centre for Mycobacteriology for MAC susceptibility testing, where the unusual morphology of the organism prompted 16S rRNA gene sequence identification. It was determined that the organism was distinct from any established mycobacterium species and was more closely related to *Mycobacterium interjectum* on the basis of 16S rDNA analysis. Two other isolates were obtained from the patient and were submitted for 16S rDNA sequencing identification. One was from sputum (strain 01-244), which was isolated earlier, in July 1999, and identified by biochemical tests as *M. gordonae*. The other strain was from thoracentesis fluid (strain 00-308, isolated September 2000). It was determined that the same organism was isolated in each case.

Strains. Three strains from the patient were made available for this study. One of these, strain 00-250^T, was sent for HPLC analysis to the Laboratoire de Santé Publique du Québec and showed a similar pattern to several isolates identified by HPLC as *M. interjectum*-like from various regions of Québec over the last decade. Sequencing of

the 16S rDNA indicated that a great proportion of these were identical to the strains isolated from the case discussed above and were included in this study to provide a better representation of the susceptibility and phenotypic and genotypic characteristics of this novel species. Strain isolation and patient data of the 16 strains (13 from Québec and three from the case report) are available as a supplementary table in IJSEM Online.

Phenotypic properties. A standard series of biochemical tests, growth temperatures and morphological examination for mycobacteriology were performed as described previously (Kent & Kubica, 1985), as listed in Table 1.

Susceptibility testing was performed using serial twofold broth microdilution as described previously (Brown *et al.*, 1992; NCCLS, 2000; Wallace *et al.*, 1986). Microdilution plates were prepared in-house using Middlebrook 7H9 broth (Difco) supplemented with 5% oleic acid/albumin/dextrose enrichment. A range of seven or eight concentrations was tested for each antimicrobial. Stock concentrations of the following antimicrobial agents were prepared from manufacturers' powders: rifampicin, ethambutol, isoniazid, amikacin and sulfamethoxazole (Sigma), ciprofloxacin and moxifloxacin (Bayer), rifabutin and linezolid (Pharmacia), streptomycin (ICN), clarithromycin (Abbott) and levofloxacin (Jansen-Otho). The 13 Québec isolates and strain 00-250^T from the case history were tested at a final inoculum of 10^3 – 10^4 c.f.u. ml⁻¹. Microdilution plates were sealed in plastic bags and incubated at 37 °C until macroscopic growth was sufficient for interpretation (7–14 days). MICs were defined as the lowest concentration of drug to inhibit visible growth, with the exception of sulfamethoxazole, for which the MIC is determined at 80% inhibition of growth compared with the growth control well. Quality control was performed using *M. avium* ATCC 700898, *Mycobacterium marinum* ATCC 927^T (Wallace *et al.*, 1986) and *M. avium* NJ-9141 (Heifets *et al.*, 1993). *Staphylococcus aureus* ATCC 29213 was used as a quality-control strain in cation-supplemented Mueller–Hinton broth when MIC ranges for mycobacteria were not available.

DNA probe. AccuProbe assays (Gen-Probe) for MAC were performed using colonies from pure culture of the 16 isolates in this study.

HPLC. HPLC by UV detection was performed according to the standard methodology (Butler *et al.*, 1996).

Sequencing of the 16S rDNA and ITS1 region. A loopful of organism from pure culture was suspended in sterile distilled water, boiled for 10 min and then subjected to mechanical lysis using a Mini Bead-Beater (Biospec Products). Lysates were then centrifuged for 2 min at 12 000 g and the supernatant was used for PCR. Amplification of the 16S rRNA gene (Rogall *et al.*, 1990; Turenne *et al.*, 2001) and the ITS1 region (Harmsen *et al.*, 2002; Turenne *et al.*, 2002) was performed as described previously and PCR products were sequenced directly using forward and reverse primers and loaded on an ABI PRISM 310 Genetic Analyzer capillary sequencer (Applied Biosystems). Sequence editing and phylogenetic analyses by CLUSTAL V (neighbour-joining method) were performed using the SEQMAN and MEGALIGN components of Lasergene 5 (DNASTAR).

Sequencing and PCR-restriction enzyme analysis (PRA) of the *hsp65* gene. A 441-bp fragment of the *hsp65* gene was amplified from the DNA lysates prepared above using the protocol described previously (Telenti *et al.*, 1993). The resulting PCR product was sequenced using the forward and reverse PCR primers TB11 and TB12. Computer-generated PRA profiles of 441-bp sequences with enzymes *Hae*III and *Bst*EII were determined using MapDraw (DNASTAR).

Table 1. Phenotypic characteristics of the study strains in comparison with other slowly growing *Mycobacterium* species

Strains/species: 1, study strains ($n=15$); 2, strain MB54784; 3, strain MCRO 8 (Springer *et al.*, 1996); 4, *M. palustre* (Torkko *et al.*, 2002); 5, *M. interjectum* 4185/92^T (Springer *et al.*, 1993; Tortoli *et al.*, 1996); 6, *M. avium* complex; 7, *M. goodii* (Kent & Kubica, 1985). Percentages of strains giving the indicated test result are given in parentheses. All species or strains grew at 25–37 °C and were negative for niacin and tolerance of 5% NaCl (not determined for *M. palustre*). ND, No data; V, variable; W+, weakly positive.

Test	1	2	3	4	5	6	7
Growth at 42 °C	– (100)	–	ND	– (100)	– (45 °C)	+	–
MAC Accuprobe	– (100)	–	ND	–	–	+	–
Pigmentation*	S (100)	S	ND	S	S	N	S
Semi-quantitative catalase	>45 (60)	<45	<45	<45 (100)	<45	<45	>45
68 °C Catalase	– (100)	–	ND	ND	–	– (76)	–
Nitrate reduction	– (100)	–	–	– (54)	–	– (92)	– (95)
Arylsulfatase (3 days/14 days)	–/– (100)	–/–	ND	– (54) (10 days)	– (3 days)	–/+ (51)	V
Tween 80 hydrolysis	– (93)	W+	+	– (100)	V	–	+
Tellurite reduction	V (53)	–	V	ND	–	– (82)	–
Growth on MacConkey agar without crystal violet	– or W+	–	ND	ND	–	– (68)	–
Urease activity	– (100)	–	–	– (100)	–	–	–
Pyrazinamidase (4 days/7 days)	– (100)/– (80)	–/–	ND	– (100)	–	– (86)/+ – (67)/+ (78)	–
β -Glucosidase	– (100)	–	ND	ND	–	ND	ND
Acid phosphatase	– (100)	–	ND	– (100)	–	ND	ND

*N, Non-chromogen; S, scotochromogen.

RESULTS AND DISCUSSION

Phenotypic properties and AccuProbe

Acid-fast microscopy revealed beaded and dispersed forms of AFB. Growth occurred best at 37 °C with colonies appearing after 2–4 weeks. On Middlebrook 7H10 solid medium, the scotochromogenic strains were initially transparent in the first 2 weeks of growth, with mature colonies showing a bright yellow pigment, most with a raised centre and a transparent, irregular apron. Photos of colonies on Middlebrook 7H10 agar are available as Supplementary Fig. A in IJSEM Online. Growth on Lowenstein–Jensen solid medium was rough and dry in texture. Strain MB54784 showed domed, smooth colonies. Biochemical test results and growth temperatures determined for each strain are indicated in Table 1. All strains, with the exception of strain MB54784 with an RLU of 1142, were positive for the MAC AccuProbe assay.

Susceptibility testing

According to current NCCLS guidelines for susceptibility testing of MAC and proposed resistance breakpoints for *Mycobacterium kansasii* (NCCLS, 2000), all isolates were resistant to isoniazid and sensitive to rifampicin, rifabutin, streptomycin, clarithromycin, amikacin, ciprofloxacin and sulfamethoxazole (Table 2). For ethambutol, 86% of isolates were susceptible using a resistance breakpoint of 10 $\mu\text{g ml}^{-1}$. If proposed breakpoints for linezolid and rapidly growing mycobacteria are employed (Wallace *et al.*, 2001), all isolates were susceptible to this antimicrobial.

Low MIC values for moxifloxacin and levofloxacin may indicate *in vitro* susceptibility to these fluoroquinolones.

Mycolic acid pattern by HPLC

With minor peak differences not affecting the overall appearance, the distinctive nine-peak pattern produced by 00-250^T, as well as of all the other isolates, belongs to the group of late-emerging, single-cluster peak patterns, such as those of *M. kansasii* and *Mycobacterium kubicae* (Fig. 1), but with more and better-developed late peaks. It is almost indistinguishable from that of the newly described *Mycobacterium palustre*, also an MAC AccuProbe-positive species (Torkko *et al.*, 2002). With its central double peak and higher end peaks, it is very similar to some published profiles of strains as well as of a Québec clinical single cluster pattern identified as *M. interjectum* (Lumb *et al.*, 1997), distinct from the double-cluster pattern of the type strain of *M. interjectum* (Butler & Guthertz, 2001).

Sequencing of the 16S rRNA gene

The nearly complete 16S rRNA gene sequence, corresponding to positions 28–1483 of the *Escherichia coli* sequence, was determined for strain 00-250^T as well as the 5' region, positions 54–510, of all subsequent isolates. All isolates were identical to 00-250^T in the 5' region, with the exception of a 1-bp mismatch in isolate MB54784. Sequencing of the rest of the 16S rDNA for this isolate revealed that no other mismatches from 00-250^T were present. The sequence contains the characteristic 12-bp omission (short helix) in the hypervariable region 18 (V3) observed in rapidly

Table 2. Antimicrobial sensitivity results of 14 strains as determined by the broth microdilution method

MIC values may be less than or equal to the concentration indicated.

Antimicrobial	Concentration range tested (µg ml ⁻¹)	Cumulative percentage of isolates for which MIC is (µg ml ⁻¹):										
		0·03	0·06	0·125	0·25	0·5	1	2	4	8	16	≥32
Amikacin	0·63–80·0						14	64	100			
Ciprofloxacin	0·125–16·0			50	79	100						
Clarithromycin	1·0–128·0						100					
Ethambutol	0·31–40·0						7	21	64	86	100	
Isoniazid	0·31–40·0											100
Levofloxacin	0·03–4·0	14		29	71	100						
Linezolid	0·5–64·0					21	79	100				
Moxifloxacin	0·03–4·0	86			93	100						
Rifabutin	0·03–4·0	100										
Rifampicin	0·06–8·0		100									
Streptomycin	0·63–80·0					64	71	86	100			
Sulfamethoxazole	2·0–256·0								21	64	93	100

growing mycobacteria, *Mycobacterium simiae* and other slowly growing species related to *M. simiae*. Its closest matches using the RIDOM database (Harmsen *et al.*, 2002) were *Mycobacterium lentiflavum* ATCC 51988 and DSM

44418^T (=ATCC 51985^T), with 5 base mismatches in the region analysed (*E. coli* positions 54–510). Analysis of the nearly complete 16S rDNA using BLAST against sequences from GenBank, EMBL or DDBJ revealed an identical match

Fig. 1. Mycolic acid patterns of strain 00-250^T (a), a clinical strain of *M. interjectum* subspecies MB51494 (b), *M. kansasii* ATCC 700732^T (c) and *M. kansasii* ATCC 12478^T (d) obtained by HPLC analysis. Peak designation is as previously suggested. HMW: High molecular mass internal standard. Strain MB51494 was identified by 16S rRNA gene sequence analysis as *M. interjectum* Patient 2 (GenBank accession no. AF014936) (Lumb *et al.*, 1997).

with *Mycobacterium* sp. MCRO 8 (X93034), with the exception of two Ns in the MCRO 8 sequence. MCRO 8 has been described as a unique species most closely resembling *Mycobacterium scrofulaceum* biochemically and was presumptively identified as a 'slowly growing species related to *M. simiae*' by 16S rDNA analysis (Springer *et al.*, 1996). Final sequence analysis was carried out using our in-house sequence database (in combination with GenBank sequences) and evaluating positions 28–1483 on the *E. coli* 16S rRNA gene (Fig. 2). Using the type strains of established species, the closest relatives were *M. interjectum* (similarity 99.1%; 13 differences), *Mycobacterium heidelbergense* (98.8%; 17), *Mycobacterium lentiflavum* (98.6%; 19), *M. simiae* (98.3%; 23) and *Mycobacterium intermedium* (98.3%; 24). An alignment of hypervariable regions A and B revealed signature sequences in the A region (Fig. 3a), with the B region being identical to that of *M. simiae*.

Sequencing of the ITS1

All strains revealed identical ITS1 sequences, with the exception of strain MB54784, which differed from the consensus sequence of all other strains by a hamming distance of 7. Neither of the sequences corresponded to ITS1 sequences in public databases. BLAST search analysis revealed closest similarity to *Mycobacterium malmoeense* (AF367025), with 91% identity in a partial overlap of 335 bases, while RIDOM analysis indicated closest similarity to *M. intracellulare* S348, with 87.54% identity in an overlap of only 281 bases.

Fig. 2. CLUSTAL W analysis of strain 00-250^T with its closest genotypic relatives. End gaps were removed to include positions 54–1470 of the *E. coli* 16S rRNA gene, with the exception of accession numbers AF014935, AF014936 and AF014937, which begin at position 110. Sequences were determined in our laboratory unless indicated by accession numbers in parentheses.

The ITS1 sequence of *M. interjectum* ATCC 51457^T revealed 83.1 and 81.0% identity against strains 00-250^T and MB54784, respectively, thereby confirming its distinction from them. An ITS1 alignment is available as Supplementary Fig. B in IJSEM Online.

Sequencing and PRA of the hsp65 gene

Sequences of a 441-bp region of *hsp65* revealed four distinct sequevars, Msk1, Msk2, Msk3 and Msk4 (Supplementary Table, Fig. 3b), most with 1 or 2 base variations from each other. The exception was strain MB54784 from Québec, or sequevar Msk4, which differed by 20 bases from Msk1. Msk1 was the most prevalent, followed by sequevar Msk2, which differed from Msk1 by 1 base, at position 411 of the 441-bp fragment. Sequevar Msk3, represented by strain MB13405 alone, differed from Msk1 by 2 bases, at positions 264 and 411 of the 441-bp fragment. The closest established species as determined by 16S rDNA analysis were also sequenced for the partial *hsp65* gene and compared with the clinical isolates (Fig. 3b). Sequevars Msk1, Msk2 and Msk3 are most closely related to *M. interjectum* ATCC 51457^T, differing from Msk1 by 7 bases. BLAST analysis of the partial *hsp65* sequence was performed with Msk1 and Msk4, using positions 46–405 of the 441-bp fragment to obtain the most accurate top score with mycobacterial *hsp65* sequences in the public database, most being either 401 or 360 bp in length. BLAST results of Msk1 revealed a top score with *M. interjectum* (AJ307632), with seven mismatches, corresponding to that determined with our in-house sequence of the type strain. BLAST results of strain MB54784 (Msk4) revealed a top score and eight mismatches with *M. gordonae* SN 601 (AF434735), which differs from *M. gordonae* ATCC 14470^T (AF434734) by 30 bases.

Computerized restriction maps of the 441-bp *hsp65* fragment as determined by MAPDRAW (LaserGene) revealed the presence of fragments of 231 and 210 bp with *Bst*EII and 127 and 145 bp with *Hae*III digestion in sequevars Msk1, Msk2 and Msk3 (Table 3). Fragments smaller than 50 bp were not taken into account. This pattern corresponds exactly with that of *M. interjectum* (da Silva Rocha *et al.*, 2002), *M. avium* III (Leao *et al.*, 1999) and MAC isolate Mav v3 (Smole *et al.*, 2002). Sequevar Msk4, however, presents a PRA pattern of 231, 131 and 79 bp fragments with *Bst*EII and 127 and 94 bp fragments with *Hae*III, which does not correspond to published PRA patterns derived from sequence analysis (Brunello *et al.*, 2001).

Comparison with possibly similar strains in the literature

Strains were not tested using the individual *M. avium* or *M. intracellulare* probes, though, with the exception of strain MB54784, all were MAC AccuProbe positive. We have conducted a literature search in an attempt to make a comparison with documented MAIX isolates and other studies examining genetic variability within MAC strains,

(a)		
(129)	(177)	(248)
TGATCTGCCCTGCACTTC	CGGATAGGACCACGGGATGCATGTCTTGT-GGT	TTGTTGGTGGGGTGACG
CA.....	TTTA.GC.....C...T-...	T.....
CA.....	A.....TTTA.GC.....C...T-...	T.....
.A.....	T.A.GC.....C.....	.A.....
.A.....	T.A.GC.....C.....	
.A.....	C.A.GC.....C...G-...	
.A.....	TTTAC.....CT..A-...	
.A.....A..	A.....T.A.GC.....C.....	
.A.....	TTTT.GC.....C...T-...	
.A.....	TT.GC.....C.....	
.A.....ACT	T.TC.GC.....C.A.GA...	
.A.....	TTTA.GC.....C...T-...	
(b)		
(644/249)	(674/279)	(806/409)
CAAGGGCGCCCAAGGAGG	GCAGATTGCGGCCACCGCAGCGATTTCGGCGGGTGACCACTCCATCGGTGACCTG	CTCGA
...TCG.....C.	...C.C.G.....C.....C.....G.....C.....	M. tuberculosis (M15467)
...TCG.....C.	...C.C.G.....C.....C.....G.....C.....	Sequevar Msk1
...TCG.....T.	...C.C.G.....C.....C.....G.....C.....	Sequevar Msk2
...CAG.....	...C.T.....G.C.C.....G.....C.....	Sequevar Msk3
G...TCG.....T.	...C.C.G.....C.....C.....G.....C.....	Sequevar Msk4
...TCG.....	...C.C.C.....G.....C.....G.....C.....	M. interjectum ATCC 51457 ^T
...TCG.....	...C.C.T.....G.C.C.....G.....C.....	M. scrofulaceum ATCC 19981 ^T
G...TCG.....	...C.C.C.....G.C.C.....G.....C.....	M. avium ATCC 25291 ^T
		M. palustre ATCC BAA-377 ^T

Fig. 3. (a) Alignment of hypervariable region A (V3) of the 16S rRNA gene. Positions indicated are according to the *E. coli* sequence. In the regions shown, *M. interjectum* Patient 3 (AF014937) is identical to the type strain. '*M. interjectum* subsp.' corresponds to sequences from GenBank designated *M. interjectum* Patient 1 (AF014935), *M. interjectum* Patient 2 (AF014936), *M. interjectum* MB739 (AJ272088) and IWGMT 90100 (X88915). All strains listed in the alignment have identical hypervariable regions B, which are therefore not included. (b) Alignment of hypervariable regions of the partial *hsp65* gene. Numbers in parentheses above the alignment represent the position within the complete *hsp65* gene in *M. tuberculosis* (M15467) (Shinnick, 1987) followed by the position within the amplified 441-bp fragment. All other sequences were determined in our laboratory.

which may or may not have been identified using DNA probes, to determine whether similar isolates have been described previously in addition to strain MCRO 8. The ITS1 sequences did not correspond to any of the 12 MAC ITS1 sequevars published previously (Frothingham &

Wilson, 1993), nor did they correspond to any ITS1 sequence in public databases.

Some studies based on PRA of the partial *hsp65* gene have shown isolates with an identical profile to that determined for our sequevars Msk1, Msk2 and Msk3. We obtained sequence data from a study that included *hsp65* sequence data (360 bp) for three strains that had an identical PRA profile to that of our strains (Smole *et al.*, 2002) and compared these with our sequences. These three strains, *M. avium* v3 920A-120 (AF241202), *M. avium* v3 121B-60.1 (AF354282) and *M. avium* v3 953A-145 (AF354283), were found to differ from our sequevar Msk1 by 15, 15 and 19 mismatches, respectively. Another study describes isolates identified as *M. avium* III, with an identical PRA pattern, that hybridized exclusively with *M. avium*-specific probes and not *M. intracellulare* (Leao *et al.*, 1999). It is unclear whether an MAC probe was done, and 16S rDNA data were not included. The proportion of *M. avium* III strains from human isolates was 6.5% (out of 107 isolates). A 441-bp *hsp65* sequence available for strain *M. avium* III (AF126033) was found to be identical to that of *M. avium* v3 953A-145 and therefore distinct from our sequences, with 96.4% sequence identity or 16 mismatches in the 441-bp fragment from Msk1.

Table 3. PRA patterns of the closest relatives of the novel isolates by *hsp65* gene sequence

Fragments below 50 bp have been omitted.

Strain	<i>Bst</i> EII	<i>Hae</i> III
Strain 00-250 ^T (and 14 other strains)	231, 210	145, 127
Strain MB54784	231, 131, 79	127, 94
<i>M. interjectum</i> ATCC 51457 ^T	231, 210	145, 127
<i>M. heidelbergense</i> ATCC 51253	231, 210	127, 112
<i>M. lentiflavum</i> ATCC 51985 ^T	441	145, 127
<i>M. simiae</i> ATCC 25275 ^T	231, 210	185, 127
<i>M. intermedium</i> ATCC 51848 ^T	231, 210	145, 129, 127
<i>M. triplex</i> ATCC 700071 ^T	325, 116	145, 127
<i>M. scrofulaceum</i> ATCC 19981 ^T	231, 210	145, 127, 95
<i>M. avium</i> ATCC 25291 ^T	231, 210	127, 103, 59
<i>M. palustre</i> ATCC BAA-377 ^T	231, 210	127, 94

Another study suggested that the *M. interjectum* PRA pattern, which we determined to be identical to that of our strains, is also similar to *M. simiae* II-3 (Taylor *et al.*, 1997), *M. intracellulare* 3 and *M. intermedium* I (da Silva Rocha *et al.*, 2002). Our computer-generated analysis of *M. intermedium* shows an additional 129 bp fragment in the *Hae*III digest, which would be indistinguishable from the 127 bp fragment using gel electrophoresis. Further PRA comparisons with available data from PRA profiles alone were difficult due to subjectivity and procedure variations, particularly with gel concentrations (Brunello *et al.*, 2001; Strässle *et al.*, 2001), where discrepancies between sequence-based PRA pattern determination and published data from gel electrophoresis (Telenti *et al.*, 1993) can reach up to 14 bases. While PRA is a common method of choice due to its ease and rapidity, the reliability of PRA pattern determination is somewhat limited and may occasionally result in misidentification (Strässle *et al.*, 2001). Finally, even within what are determined to be isolates of the MAC, various PRA patterns are observed, some of which are similar to those of other NTM species.

We recognized a similarity between our isolates and strain FI-16795 (Tortoli *et al.*, 1997) due its scotochromogenicity, MAC AccuProbe positivity and similar HPLC profile. This isolate was subsequently identified as *M. palustre* and was evaluated, along with other isolates of *M. palustre*, in the publication describing the species (Torkko *et al.*, 2002).

Clinical significance

The patient described in this case history presented with a clinical history of bronchiectasis of unknown aetiology, documented first in around 1990 by CT. The absence of an alternative pathogenic agent supports the potential clinical relevance of this novel species. It is likely that this patient developed bronchiectasis from recurrent infections or an underlying lung disease, which rendered her susceptible to an atypical mycobacteriosis caused by this organism. It is well known that most NTM are not considered pathogenic; however, correct species determination is important in order to make appropriate treatment decisions. The limitations of biochemical testing were demonstrated in this case, as the NTM recovered initially from the patient was identified as *M. gordonae* and considered not to be responsible for the disease, even though the recovery of AFB from three sputum samples, according to the American Thoracic Society criteria (Wallace *et al.*, 1997), would suggest a diagnosis of an NTM infection. Perhaps earlier intervention, in 1999, would have prevented further lung damage. All other isolates in our study were recovered from respiratory specimens, and no extensive clinical history is available. However, in two instances, the same isolate was recovered twice from the same patient in two different years. Also, one patient tested repeatedly from 1993 to 1996 yielded seven isolates, and the organism's pathogenicity in this case is therefore considered highly probable (Wallace *et al.*, 1997).

Differentiation of *Mycobacterium saskatchewanense* sp. nov. from other slowly growing NTM

The group of unique strains evaluated in this study represent a species that, depending on the methodology used for identification, mimics various established species of mycobacteria. Without sequence-based identification, *M. saskatchewanense* sp. nov. may be difficult to recognize. Although the MAC AccuProbe is positive for these isolates, colony morphology and pigmentation are very distinct from *M. avium* or *M. intracellulare*. Additionally, the inability to grow at 42 °C, a positive Tween 80 hydrolysis test and negative pyrazinamidase test differentiate this species from *M. avium* and *M. intracellulare*. *M. saskatchewanense* can also be differentiated from another recently described AccuProbe positive species, *M. palustre*, by its inability to grow at 42 °C and negative results for urease, pyrazinamidase and acid phosphatase. Biochemically, *M. saskatchewanense* most closely resembles *M. gordonae*, and phenotypic differentiation is subtle. This redundancy in biochemical profiles is not surprising, given the large number of mycobacteria currently established (>100). The HPLC pattern of the strains evaluated in this study is distinct from the other late-emerging single-cluster peak patterns, with the exception of *M. palustre*, mainly in the relative heights of corresponding late peaks. The HPLC pattern is also very similar to some published profiles of strains identified as *M. interjectum*; however, it is distinct from the pattern observed in the type strain of *M. interjectum*. Despite sequence mismatches in the *hsp65* gene, PRA analysis of *M. saskatchewanense*, *M. interjectum*, selected strains designated MAC and *M. intermedium* using enzymes *Bst*EII and *Hae*III revealed an identical pattern. While the type strain of *M. intermedium* is non-chromogenic, the morphologies of *M. interjectum* and *M. saskatchewanense* are similar. *M. saskatchewanense* can be differentiated from *M. interjectum*, MAC, *M. gordonae*, *M. kubicae*, *M. palustre* and other mycobacteria by its 16S rRNA gene, ITS1 and *hsp65* gene sequences, confirming the isolate as representing a novel species.

Description of *Mycobacterium saskatchewanense* sp. nov.

Mycobacterium saskatchewanense (sas.kat.che.wa.nen'se. N.L. neut. adj. *saskatchewanense* pertaining to Saskatchewan).

Cells are acid-alcohol-fast, long, beaded rods. Grows at 25–37 °C, but not at 42 °C. The organism shows a bright yellow pigment under both light and dark conditions. Strains with an identical 16S rRNA gene sequence to that of 00-250^T are MAC AccuProbe positive. Negative for acid phosphatase, arylsulfatase, β -glucosidase, niacin, nitrate reductase, pyrazinamidase and urease and does not grow on 5% NaCl. Some strains show a thin film of growth on MacConkey agar without crystal violet. Other variable test results include semi-quantitative catalase and tellurite reduction. Most isolates are strongly positive for Tween 80

hydrolysis and all isolates have heat-stable catalase activity. Mycolic acids of the cell wall produce a single-cluster pattern by HPLC, typical of *M. kubicae*, *M. palustre* and some documented clinical strains of *M. interjectum*. Sequencing of the 16S rRNA, *hsp65* gene and ITS1 region reveals unique data that are crucial in the differentiation of this species from selected NTM species. Isolates are susceptible *in vitro* to amikacin, clarithromycin, ciprofloxacin, linezolid, levofloxacin, moxifloxacin, rifabutin, rifampicin, streptomycin and sulfamethoxazole and are resistant to isoniazid. Eighty-six per cent of isolates are susceptible to ethambutol, with a resistance breakpoint of 10 µg ml⁻¹. Strains have been isolated from human respiratory samples and were proven to be clinically significant in our case study. Strain MB54784 may constitute a distinct species, since it shows significant *hsp65* and ITS1 sequence variation; however, due to phenotypic and 16S rDNA similarities, this is difficult to establish at this time.

The type strain is strain 00-250^T (=ATCC BAA-544^T = DSM 44616^T = CIP 108114^T), which was isolated from the sputum of a patient with bronchiectasis.

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