Mycobacterium minnesotense sp. nov., a photochromogenic bacterium isolated from sphagnum peat bogs

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Several intermediate-growing, photochromogenic bacteria were isolated from sphagnum peat bogs in northern Minnesota, USA. Acid-fast staining and 16S rRNA gene sequence analysis placed these environmental isolates in the genus Mycobacterium, and colony morphologies and PCR restriction analysis patterns of the isolates were similar. Partial sequences of hsp65 and dnaJ1 from these isolates showed that Mycobacterium arupense ATCC BAA-1242T was the closest mycobacterial relative, and common biochemical characteristics and antibiotic susceptibilities existed between the isolates and M. arupense ATCC BAA-1242T. However, compared to nonchromogenic M. arupense ATCC BAA-1242T, the environmental isolates were photochromogenic, had a different mycolic acid profile and had reduced cell-surface hydrophobicity in liquid culture. The data reported here support the conclusion that the isolates are representatives of a novel mycobacterial species, for which the name Mycobacterium minnesotense sp. nov. is proposed. The type strain is DL49T (=DSM 45633T = JCM 17932T = NCCB 100399T).

Unlike Mycobacterium tuberculosis, non-tuberculous mycobacteria (NTM) are not obligate pathogens and can be found in a number of different environmental habitats (Falkinham, 2009, 2010; Slany et al., 2010). In recent years, NTM have become an increasing threat to humans as opportunistic infectious agents (Cook, 2010; Cassidy et al., 2009). Among the newly discovered and potentially pathogenic NTM is Mycobacterium arupense (Cloud et al., 2006), which is a member of the Mycobacterium terrae complex of mycobacteria. Although M. arupense has a mycolic acid profile similar to Mycobacterium nonchromogenicum, the two species differ in growth temperature range, drug sensitivities, speed of growth, and gene sequences (Cloud et al., 2006). Since its discovery, M. arupense has been isolated from a wide variety of sources including sputum samples (Masaki et al., 2006; Neonakis et al., 2010) as well as sterile sites in the body (Pauls et al., 2003), a Korean river and a tap water source (Lee et al., 2008), the River Seine in France (Radomski et al., 2010), small terrestrial mammals of Tanzania and the Czech Republic (Durnez et al., 2008; Slany et al., 2010) and from several diverse environmental samples including refrigerated dog food and soils from an eagle’s nest and duck houses (Martin et al., 2010; Slany et al., 2010). Knowing the phenotypic diversity and environmental reservoirs of M. arupense is important since the bacterium is a potential human pathogen. During a recent survey of mycobacteria from sphagnum peat moss bogs of northern Minnesota, we repeatedly cultured bacteria that had partial 16S rRNA gene sequences similar to M. arupense ATCC BAA-1242T. Thirteen of these isolates were characterized using a polyphasic approach to resolve their taxonomic positions. Although the closest identified relative of these isolates was M. arupense ATCC BAA-1242T, enough different features existed to suggest the isolates are members of a novel species of the genus Mycobacterium. While slight variations existed among the colony morphologies of the 13 environmental isolates, strain DL49T was designated the type strain.

Water, soil and sphagnum peat samples were collected from three sphagnum peat bogs in the northern Minnesota region in October 2009 (Fig. S1, available in IJSEM Online). The collection sites were the Big Bog State Recreation Area (48.82 N, 94.58 W), the Savanna Portage State Park (46.82 N, 93.15 W) and a bog near Duluth (46.77 N, 92.12 W). Bog samples were collected at various...
depths in the moss, including the grey decomposition layer and underlying water within the bogs. Mycobacterial isolates were obtained from the samples using a modification of a protocol described by Chilima et al. (2006). Briefly, bacterial and fungal spores were allowed to germinate into vegetative states by suspending 2 g bog sample in 5 ml nutrient broth (Difco) and shaking at 150 r.p.m., 37 °C for 2 h. Insoluble material was removed, the liquid cultures were centrifuged (3000 g, 15 min) and cell pellets were resuspended in 11 ml mycobacterial selection buffer (containing ml⁻¹: 115 µg malachite green, 45 µg cycloheximide, 3.6 mg NaOH). Cells were incubated at room temperature for 20 min before adding 10 ml of 100 mM phosphate buffer (pH 6.9) to neutralize samples. Cells were concentrated 100-fold by centrifugation (3000 g, 15 min) and spread onto Middlebrook 7H11 plates (Difco) containing (ml⁻¹) 250 µg cycloheximide and 17.5 µg nalidixic acid, with or without oleic acid, albumin, dextrose, catalase (OADC; Becton Dickinson) for enrichment. Plates were incubated at either 28 °C or 37 °C and examined for colonies at 3, 7 and 14 days post inoculation and then every 2 weeks for up to 8 weeks. Resulting colonies were subcultivated and subjected to Gram staining and Ziehl–Neelsen acid-fast staining (Beveridge et al., 2007). The presence of OADC in the 7H11 agar plate medium greatly increased the number of resulting mycobacterial candidates and growth at 28 °C typically produced 2–3-fold more mycobacterial colonies than growth at 37 °C. A total of 720 acid-fast isolates were saved and designated strains DL1–DL720. Approximately 65% of these isolates were ‘fast-growing’ and capable of forming colonies within 7 days. ‘Intermediate-growing’ isolates produced visible colonies by 7–14 days and ‘slow-growing’ isolates typically took longer to form colonies.

The acid-fast-staining mycobacterial candidates were first grouped according to rate of growth, optimal growth temperature and colony pigmentation and morphology. Colonies were suspended in phosphate-buffered saline and lysed using 0.1 mm diameter glass beads and a FastPrep FP120 bead-beating device (ThermoSavant). PCR restriction analysis (PRA) of hsp65 amplicons (411 bp) was performed using BstEII and HaeIII, as previously described (Telenti et al., 1993). In addition, the restriction enzyme CfoI was used, as previously described (Vaneechoutte et al., 1993). The PCR pattern of the 411-bp hsp65 PCR product from M. arupense has been previously reported (Neonakis et al., 2010) with two expected fragments from BstEII digestion (115 and 325 bp) and four expected fragments from HaeIII digestion (40, 50, 60 and 145 bp). Fig. S2a shows the PRA patterns of the hsp65 PCR product from M. arupense ATCC BAA-1242T using the three restriction enzymes. A 325-bp fragment was visible for the BstEII digestion and a 145 bp band was seen for the HaeIII digestion. CfoI digestion would be expected to produce five fragments, with the largest two being 102 bp and 224 bp, and bands for both these sizes were observed following CfoI digestion (Fig. S2a). PRA was performed on 112 intermediate-growing bog mycobacterial candidates, which were then grouped based upon similarities of PRA patterns. 29 environmental isolates had PRA patterns identical to that of M. arupense ATCC BAA-1242T, and the patterns for 13 of these isolates are shown in Fig. S2b–d.

In addition to hsp65, PCR was used to amplify the 16S rRNA gene (rrs; Mendum et al., 2000) and dnaJ1 (Yamada-Noda et al., 2007), and the amplification products were sequenced at the University of Minnesota Biomedical Genomics Center. Concatenated DNA sequences were constructed (rrs–dnaJ1–hsp65) and the evolutionary history between isolates was inferred using the neighbour-joining method (Saitou & Nei, 1987). Phylogenetic analyses were conducted using MEGA4 software (Tamura et al., 2007). Bootstrap values were determined for 1000 replicates (Felsenstein, 1985) and the evolutionary distances were computed using Kimura’s two-parameter method (Kimura, 1980). Positions containing alignment gaps and missing data were eliminated only for pairwise sequence comparisons (pairwise deletion option). In addition to M. arupense ATCC BAA-1242T, other members of the M. terrae complex were included in the phylogenetic tree as well as M. tuberculosis ATCC 27294T and Nocardioides farcinica IFM 10152 which was used as an outgroup. The numbers of nucleotide mismatches and sequence similarities between M. arupense ATCC BAA-1242T and the 13 environmental isolates are shown in Table S2. The 13 environmental isolate sequences were closely related to M. arupense ATCC BAA-1242T but grouped in a separate cluster that was designated the ‘DL49 cluster’ (Fig. 1).

To observe cultural characteristics, 20-µl aliquots of the 13 environmental isolates and M. arupense ATCC BAA-1242T grown in 7H9 media with Tween 80 (0.05%, v/v) and enriched with albumin, dextrose, catalase (ADC; Becton Dickinson) were spotted onto 7H11 agar enriched with OADC. Additionally, serial dilutions of liquid cultures were plated to obtain isolated colonies. Plates were incubated at 30 °C for 2 weeks in the dark and then transferred to a bench top with ambient light for an additional week. Spot cultures and isolated colonies were photographed and compared with M. arupense ATCC BAA-1242T (Fig. S3). While M. arupense ATCC BAA-1242T was nonchromogenic, 12 of the 13 environmental isolates were photochromogenic and produced pigments ranging from orange to pinkish-orange. All but one environmental isolate grew optimally at 30 °C, with little or no growth at 37 °C. The one exception was isolate DL685, which grew equally fast at both temperatures.

M. arupense ATCC BAA-1242T and the 13 environmental isolates were subjected to the following biochemical tests, as previously described (Leao et al., 2004): nitrate reductase, urease, Tween 80 hydrolysis, 3-day and 14-day arylsulfatase and tolerance to 5% NaCl. The 68 °C catalase test was performed, as previously described (Tierno & Milstoc, 1981). All cultures were grown at 30 °C prior to tests. Results are shown in Table 1. With only three
exceptions (strains DL189, DL630 and DL678A), the environmental isolates had biochemical properties identical to *M. arupense* ATCC BAA-1242T.

Antibiotic susceptibilities of *M. arupense* ATCC BAA-1242T and strain DL49T were compared using (μg per disc unless otherwise stated) penicillin (10 U), ampicillin (10), trimethoprim (5), chloramphenicol (30), tetracycline (30), streptomycin (10), sulfinpoxide (0.25 mg), bacitracin (10 U), neomycin (30), erythromycin (2), ciprofloxacin (5), gentamicin (10), and clindamycin (2) discs (Becton Dickinson) and 7H11 OADC as the basal medium. Zones of inhibition were measured at 6 and 12 days. Both strains showed complete resistance to gentamicin, clindamycin, erythromycin, trimethoprim, tetracycline, bacitracin and chloramphenicol and equivalent partial sensitivities to neomycin and ampicillin. Strain DL49T showed partial susceptibility to ciprofloxacin while *M. arupense* ATCC BAA-1242T showed no susceptibility. Conversely, *M. arupense* ATCC BAA-1242T was very sensitive to penicillin, DL150B, DL215A, DL477 and DL685 by using HPLC and the Microbial Identification system (MIDI). The mycolic acid profiles of *M. arupense* ATCC BAA-1242T and strain DL49T are given in Fig. 2, and of all strains tested in Fig. S4. While *M. nonchromogenicum* was reported to have an identical mycolic acid profile to *M. arupense* (Cloud et al., 2006), all five environmental isolates tested here had a unique cluster of peaks not seen in *M. arupense* ATCC BAA-1242T (cluster 2 in Fig. 2b and Fig. S4). Differences in mycolic acids may be the cause of the extreme hydrophobicity of *M. arupense* ATCC BAA-1242T observed in 7H9 liquid cultures enriched with OADC containing 0.05 % (v/v) Tween 80, in contrast to the dispersion of environmental isolate cells in liquid culture (Fig. S5).

Kazda (2000) reported that mycobacterial species are diverse in sphagnum peat moss worldwide and Cassidy et al. (2009) observed that opportunistic NTM infections can arise from exposure to peat in planting soil. In our sampling of three northern Minnesota peat bogs, we found numerous mycobacterial isolates with identical PRA patterns to *M. arupense* ATCC BAA-1242T. Phylogenetic analysis based upon partial 16S rRNA gene, *dnaJ1* and *his65* sequences indicated that the 13 isolates are genetically distinct from *M. arupense* ATCC BAA-1242T. Taken together, the distinctions in DNA sequences, the unique HPLC profiles of mycolic acids, the light-induced pigment production and the lack of differences in hydrophobicity in liquid culture supports the conclusion that the isolates are members of a mycobacterial species distinct from *M. arupense*, for which the name *Mycobacterium minnesotense* sp. nov. is proposed. Strain DL49T has been selected to represent the type strain, but spot-culture morphologies alone indicate a natural variation among members of this species in collections of mycobacteria retrieved from Minnesota bogs (Fig. S3).

### Description of *Mycobacterium minnesotense* sp. nov.

*Mycobacterium minnesotense* (min.ne.so.ten’s.e. N.L. neut. adj. minnesotense of or belonging to Minnesota).
**Mycobacterium minnesotense** sp. nov.

Gram-positive, acid-fast-positive, non-spore-forming, non-motile curved or straight rods. Smooth, pinkish-orange, photochromogenic colonies appear after 7–10 days on 7H11 agar enriched with OADC. Grows at 28–34°C with optimal growth at 30°C. No growth at 37°C or on 7H11 plates without OADC. No growth on MacConkey agar without crystal violet. Grows with 5% (w/v) NaCl. Positive reaction in tests for Tween 80 hydrolysis, 68°C catalase and 14-day arylsulfatase activity, but negative reaction in tests for nitrate reductase, urease and 3-day arylsulfatase activity. The type strain is resistant to gentamicin, clindamycin, erythromycin, tetracycline, bacitracin, chloramphenicol, streptomycin, penicillin and sulfisoxazole but is susceptible to neomycin, ampicillin and ciprofloxacin. Genetically, 16S rRNA gene, *dnaJ1* and *hsp65* sequences are most similar to *Mycobacterium arupense* ATCC BAA-1242T, and PRA patterns are identical to *M. arupense* ATCC BAA-1242T. The mycolic acid profile of the type strain is similar to *M. arupense* ATCC BAA-1242T but contains two unique peaks that distinguish it from *M. arupense* ATCC BAA-1242T. Pinkish-orange pigmentation, dispersion in 7H9 liquid culture with OADC and resistance to penicillin, streptomycin and sulfisoxazole can be used to differentiate this species from the closely related species *M. arupense* ATCC BAA-1242T.

The type strain is DL49T (=DSM 45633T=JCM 17932T =NCCB 100399T), isolated from a sphagnum peat bog in northern Minnesota, USA.

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**References**

Mycobacterium arupense in Japan: novel slowly growing, nonchromogenic clinical isolate from clinical specimen and the first isolation report of mycobacteria in the human environment.


