Mycobacterium nebraskense sp. nov., a novel slowly growing scotochromogenic species

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The characterization of a novel slowly growing, scotochromogenic *Mycobacterium* species is reported. This previously undescribed mycobacterial species was isolated from five different patients with symptomatic pulmonary infections. All isolates were acid-fast-positive and the mycolic acid profiles were unique and supported placement into the genus *Mycobacterium*. Phenotypic characteristics of each strain included optimal growth after 3 weeks at a temperature range of 30–35 °C, yellow pigmentation after incubation in the dark and production of a heat-stable catalase. The 16S rRNA gene and internal transcribed spacer 1 sequences were identical for all five strains, but distinct from all known mycobacterial species. Phylogenetic analysis based on the 16S rRNA gene sequence placed the novel species within the slowly growing mycobacteria group in close proximity to *Mycobacterium malmoense*, *Mycobacterium avium*, *Mycobacterium kansasii* and *Mycobacterium scrofulaceum*. These data support the conclusion that the related five described organisms represent a novel *Mycobacterium* species, for which the name *Mycobacterium nebraskense* sp. nov. is proposed, with the type strain UNMC-MY1349^T (=ATCC BAA-837^T =DSM 44803^T).

Mycobacterium species are common causes of pulmonary infections in both humans and animals (Falkinham, 1996). Although members of the *Mycobacterium tuberculosis* complex are the most frequent species to cause pulmonary infection worldwide, many non-tuberculous *Mycobacterium* (NTM) species have been found capable of causing similar infections (El-Solh *et al.*, 1998; Koh *et al.*, 2002).

The differential identification of *Mycobacterium* species is important for the control and treatment of infection. Currently, most clinical laboratories use conventional phenotypic assays to identify suspected mycobacterial pathogens. These assays were developed primarily for the identification of the more common clinically associated mycobacterial species and frequently are unable to identify uncommon or newly described *Mycobacterium* species due to the lack of discriminatory power (Kirschner *et al.*, 1993; Tortoli *et al.*, 1999).

Abbreviations: ITS, internal transcribed spacer; NTM, non-tuberculous *Mycobacterium*; SI, similarity index.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and ITS1 sequences of *M. nebraskense* UNMC-MY1349^T are AY368456 and AY368458, respectively.

An alignment of hypervariable regions of the 16S rRNA gene and the ITS1 of the novel species and related mycobacteria and a fuller phylogenetic tree are available as supplementary material in IJSEM Online.

To overcome the limitation of phenotypic assays, genetic methods of analysis have been developed (Roth *et al.*, 2000). The combination of molecular assays and conventional methods creates a conclusive identification approach that can provide for the identification of unusual mycobacterial species and also for the description of novel taxa (Floyd *et al.*, 2000). In the present study, a novel NTM species isolated from five different patients with general symptoms of pulmonary infections is described.

Five acid-fast-positive isolates from sputum previously identified as *Mycobacterium* species were submitted to our regional laboratory for species identification (strains identified as UNMC-MY1349^T, UNMC-MY1293, UNMC-MY1174, UNMC-MY2873 and UNMC-MY3257). These isolates were collected from individual patients representing multiple geographical regions throughout Nebraska. A lung biopsy sample that was collected and archived at –70 °C for further analysis was also obtained.

Bacterial cell morphology for each isolate was determined by acid-fast staining using the Ziehl–Neelsen method and by the auramine O fluorescent stain method. Colony morphology, pigment production under dark and light conditions and ability to grow at temperatures ranging from 25 to 42 °C were examined following inoculation to both Lowenstein–Jensen (LJ) and Middlebrook 7H11 media. Conventional biochemical testing was performed

as described previously by Kent & Kubica (1985). Tests included niacin accumulation, nitrate reductase, arylsulfatase on day 3, semi-quantitative catalase (45 mm), 68 °C catalase, Tween 80 hydrolysis, urease activity, tolerance to 5 % sodium chloride, growth on MacConkey agar without crystal violet and pyrazinamidase production.

HPLC mycolic acid analysis using procedures as described by Miller (1997) was performed on each isolate following growth on Middlebrook 7H10 medium. An internal calibration standard spiked with low- (LMIS) and high-molecular-mass internal standards (HMIS) (Ribi Immunochem) was included with each HPLC run. The mycolic acid compositions of the isolates were matched with those in a library of known mycobacterial profiles and expressed numerically using a similarity index (SI). The SI expresses how closely the mycolic acid composition of an unknown isolate matches those profiles within the library. An exact match of the mycolic acid of an unknown with the mean of a library profile results in an SI of 1·000. As each mycolic acid varies from the mean percentage, the SI decreases in proportion to the cumulative variance between the composition of the unknown and the library entry.

Genomic DNA was extracted from cultured isolates by the glass bead agitation method as described previously by Plikaytis *et al.* (1990). The lung tissue biopsy was homogenized using a Brinkmann Polytron P10/35 homogenizer (Diversified Equipment Company, Inc.) prior to extraction of the DNA using the glass bead agitation method. The crude DNA extract was purified using the QIAmp blood kit (Qiagen) according to protocols provided by the manufacturer. Two different DNA complex genetic targets were analysed to include the 16S rRNA gene and the internal transcribed spacer 1 (ITS1) region located between the 16S and 23S rRNA genes. The complete 16S rRNA gene (1506 bp) was amplified by PCR as described by Turenne *et al.* (2001) and the hypervariable region of the ITS1 target (209 bp) was amplified as described by Roth *et al.* (2000). PCR products of all targets were cloned directly following amplification using the standard protocol of the Original TA cloning kit (Invitrogen). Purified plasmids from selected colonies were screened for the correct insert by digestion with endonuclease *EcoRI* (New England Biolabs) and analysed by gel electrophoresis for recovery of the approximately sized product. Selected plasmids were sequenced at the Eppley Molecular Biology Core Laboratory (University of Nebraska Medical Center) using the universal M13 forward and reverse sequencing primers. The analysis was repeated three times for each of the five isolates from separate single colonies to ensure the accuracy of the generated sequences. The 16S rRNA gene and ITS1 sequences of each studied isolate were compared with the GenBank information using the BLAST analysis program (<http://www.ncbi.nlm.nih.gov/blast/>).

Multiple sequence alignments of the type strain and reference 16S rRNA gene sequences of a wide range of both slowly and rapidly growing mycobacterial species were

created using the PILEUP program from the GCG Wisconsin Package for Sequence Analysis (Accelrys Inc.). Regions of uncertain alignment were truncated from each end of the gene. Evolutionary analysis of the aligned sequences was performed using PHYLIP version 3.5c. A tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). The analysis included calculation of the distance matrix using DNADIST that was applied to distances corrected for multiple hits and for unequal transition and transversion rates according to Kimura's two-parameter model (Kimura, 1980). Due to the high similarity of mycobacterial 16S rRNA genes, bootstrapping was not applied. However, the tree positions were confirmed by parsimony analysis for generation of a majority rule consensus tree using CONSENSE. The tree was rooted with *Nocardia asteroides* as an outgroup.

The results showed that all five strains were acid-fast-positive, non-motile and non-spore-forming bacilli. Mature colonies developed at 3 weeks on Middlebrook 7H11 medium and required 4 or more weeks on LJ medium to mature. The growth temperature range was 25–35 °C with optimal growth obtained at 30–35 °C and no growth at 42 °C. Mature colonies were rough in appearance with an elevated centre and yellow pigmentation under both dark and photoinduction conditions. No growth occurred on MacConkey agar without crystal violet or by the addition of 5 % NaCl to the culture medium.

All five strains were negative for nitrate reductase, urease activity, arylsulfatase activity, niacin accumulation, semi-quantitative catalase and pyrazinamidase production and positive for heat stable catalase (Table 1). The five unknown strains showed variable results for Tween 80 hydrolysis. The phenotypic characteristics of the five unknown strains did not match those of any of the other slowly growing mycobacterial species.

HPLC analysis of mycolic acid content showed identical patterns for all five strains. The mycolic acid profile of the unknown was characterized by a widely separated double peak clusters pattern with prominent peaks in the early cluster (Fig. 1). This pattern did not match any of the previously reported mycobacterial mycolic acid profiles (Butler & Guthertz, 2001). The closest match was with the *Mycobacterium intracellulare-avium-scrofulaceum* complex, with an SI of 0·552. The most significant qualitative difference was apparent in peak number one, which eluted before the LMIS in the unknown profile and after the LMIS in the *M. intracellulare-avium-scrofulaceum* complex. A quantitative difference between the two profiles was also apparent in the different peak numbers and heights.

A BLAST search of the GenBank database using the partial ITS1 sequence gave the closest match (96 %) to both *Mycobacterium avium* complex and to *Mycobacterium* sp. RiVM9900327. When conducting a BLAST search of the GenBank database using the complete 16S rRNA gene sequence, the closest match was to an unknown

Table 1. Physiological characteristics that differentiate strain UNMC-MY1349^T from other closely related slowly growing *Mycobacterium* species

Species/strains: 1, UNMC-MY1349^T; 2, *M. kansasii*; 3, *M. avium* complex; 4, *M. scrofulaceum*; 5, *M. malmoense*; 6, *M. szulgai*; 7, *M. bohemicum*; 8, IWGMT 90160. +, Positive; –, negative; V, variable. Selective data for all known species were taken from Kubica *et al.* (1975), Kent & Kubica (1985) and Wayne *et al.* (1996). Strains UNMC-MY1349^T, UNMC-MY1293, UNMC-MY1174, UNMC-MY2873, and UNMC-MY3257 all had identical biochemical patterns. All species/strains are negative for niacin accumulation.

Phenotypic test	1	2	3	4	5	6	7	8
Growth at:								
25 °C	+	+	+	+	+	+	V	+
42 °C	–	–	V	–	V	–	–	–
Pigment production*	S	P	N	S	N	S/P	S	S
Nitrate reduction	–	+	–	–	–	+	–	–
Tween 80 hydrolysis (at 10 days)	V	+	–	–	+	V	–	–
Activity for:								
Arylsulfatase (at 3 days)	–	–	–	V	–	V	–	–
Catalase (semi-quantitative at >45 mm)	–	+	–	+	–	+	–	+
Catalase (heat-stable at 68 °C for 20 min)	+	+	V	+	V	+	V	+
Urease	–	+	–	+	V	+	+	–
Pyrazinamidase production	–	–	+	V	+	+	+	+

*N, Non-photochromogenic; P, photochromogenic; S, scotochromogenic.

Mycobacterium sp. (IWGMT 90160). It was interesting to note that a BLAST analysis of the first 500 bases of the 16S rRNA gene gave 99% similarity to four previously unrecognized *Mycobacterium* sp. (strain N097, accession no. AY215318; strain N109C, AY215208; strain N199, AY215214; and strain N179, AY215211). It would be interesting to evaluate the ITS1 sequences of these isolates to determine similarity to our five strains. Supplementary

Fig. A available in IJSEM Online shows a comparison of the hypervariable region A and B of the 16S rRNA gene and the hypervariable region of the ITS1 target of UNMC-MY1349^T with those of selected slow-growing mycobacteria.

A phylogenetic tree was created to show the position of the novel species with regard to both slowly and rapidly growing mycobacteria using the entire 16S rRNA gene sequence (Fig. 2). The result of the phylogenetic analysis grouped strain UNMC-MY1349^T within the slowly growing *Mycobacterium* species, close to *Mycobacterium kansasii*

Fig. 1. Mirror-image comparison between the HPLC patterns of mycolic acid content for *M. nebraskense* sp. nov. (upper trace) and *M. intracellulare-avium-scrofulaceum* complex (lower trace). Represented on each profile are the low-molecular-mass internal standard (LMIS) and the high-molecular-mass internal standard (HMIS).

Fig. 2. Phylogenetic tree of selected *Mycobacterium* species using the entire 16S rRNA gene sequence. The tree was constructed using the neighbour-joining method and rooted with *Nocardia asteroides* as an outgroup. GenBank accession numbers are shown in parentheses.

and *Mycobacterium gastri*. This unknown species was grouped closest to the uncharacterized *Mycobacterium* sp. IWGMT-90160. A fuller phylogenetic tree is available as Supplementary Fig. B in IJSEM Online.

The distinct mycobacterial sequence characteristics of two different targets together with the uniqueness of the mycolic acid profile confirm the unknown isolate as representing a novel mycobacterial species rather than a variant strain of a previously described species. The isolation of the unknown species from the sputum of five pulmonary infected patients in this study suggested a likely causative association between infection with this unknown species and pulmonary disease.

Description of *Mycobacterium nebraskense* sp. nov.

Mycobacterium nebraskense (ne.bras.ken'se. N.L. neut. adj. *nebraskense* referring to the State of Nebraska, USA).

Cells are non-spore-forming rods that stain acid-fast-positive. Mature growth is produced in 3 weeks on Middlebrook 7H11 medium and in 4 or more weeks on LJ medium at 25–35 °C, with optimal growth at 30–35 °C and no growth at 42 °C. Mature colonies are rough with an elevated centre and produce strong yellow pigmentation in the dark. The organism is positive for heat-stable catalase and negative for nitrate reductase, urease activity, niacin accumulation, arylsulfatase activity at 3 days and pyrazinamidase production. Less than 45 mm foam is produced in the semi-quantitative catalase test and variable results are indicated for Tween 80 hydrolysis. Growth is inhibited on MacConkey agar without crystal violet and by the addition of 5 % NaCl to the culture medium. Exhibits a unique mycolic acid profile by HPLC analysis characterized by a widely separated double peak clusters pattern that is typical for genus *Mycobacterium*. Genetically, the organism has unique *Mycobacterium* sequences for the 16S rRNA gene and ITS1 region targets. Phylogenetic analysis using 16S rRNA gene sequences shows that *M. nebraskense* belongs to the slowly growing mycobacteria and is closely related to *M. kansasii*, *M. scrofulaceum*, *Mycobacterium malmoeense* and *M. avium*. The sequences of the 16S rRNA gene (AY368456) and the partial ITS1 region (AY368458) of the type strain have been deposited in GenBank.

Isolated from human sputum in Nebraska, USA. The type strain is UNMC-MY1349^T (=ATCC BAA-837^T=DSM 44803^T).

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