Mycobacterium talmoniae sp. nov., a slowly growing mycobacterium isolated from human respiratory samples

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Abstract
A novel slowly growing, non-chromogenic species of the class Actinobacteria was isolated from a human respiratory sample in Nebraska, USA, in 2012. Analysis of the internal transcribed spacer sequence supported placement into the genus Mycobacterium with high sequence similarity to a previously undescribed strain isolated from a patient respiratory sample from Oregon, USA, held in a collection in Colorado, USA, in 2000. The two isolates were subjected to phenotypic testing and whole genome sequencing and found to be indistinguishable. The bacteria were acid-fast stain-positive, rod-shaped and exhibited growth after 7–10 days on solid media at temperatures ranging from 25 to 42°C. Colonies were non-pigmented, rough and slightly raised. Analyses of matrix-assisted laser desorption ionization time-of-flight profiles showed no matches against a reference library of 130 mycobacterial species. Full-length 16S rRNA gene sequences were identical for the two isolates, the average nucleotide identity (ANI) between their genomes was 99.7% and phylogenetic comparisons classified the novel mycobacteria as the basal most species in the slowly growing Mycobacterium clade. Mycobacterium avium is the most closely related species based on rpoB gene sequence similarity (92%), but the ANI between the genomes was 81.5%, below the suggested cut-off for differentiating two species (95%). Mycolic acid profiles were more similar to M. avium than to Mycobacterium simiae or Mycobacterium abscessus. The phenotypic and genomic data support the conclusion that the two related isolates represent a novel Mycobacterium species for which the name Mycobacterium talmoniae sp. nov. is proposed. The type strain is NE-TNMC-100812T (=ATCC BAA-2683=DSM 46873T).

Non-tuberculous mycobacteria (NTM) are primarily environmental organisms with more than 170 officially recognized species [1]. Many mycobacterial species have been isolated from human samples and are thought to be associated with human disease. The most common methods for NTM species identification include molecular approaches such as sequencing of the 16S rRNA, internal transcribed spacer (ITS) or other informative housekeeping genes such as rpoB and hsp65 [2–4]. These methods, along with whole genome sequencing, have enabled the characterization of a large number of new NTM species in recent years [1]. Robust species identification within the genus Mycobacterium is important for the description of potential human pathogens, epidemiological analysis and treatment of infection. In the present study, the same novel NTM species was isolated 12 years apart from the respiratory tracts of two separate individuals from distinct geographical regions in the USA.

In 2000, an unidentified acid-fast bacillus (AFB) was isolated from a sputum sample of a patient referred to the National Jewish Health in Denver, Colorado, from her home in Oregon. The ITS region was sequenced and the isolate was given the unofficial name ‘Mycobacterium coloregonium’ and was deposited with the American Type Culture Collection (ATCC) in

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Abbreviations: AFB, acid-fast bacillus; ANI, average nucleotide identity; AST, antimicrobial susceptibility testing; ITS, internal transcribed spacer; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; NTM, non-tuberculous mycobacteria.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, rpoB and hsp65 gene sequences of strain NE-TNMC-100812T are KX008970, KX008971 and KY30182, respectively.

One supplementary figure is available with the online Supplementary Material.
2004 under the name ATCC BAA-1052. This species name, however, has not yet been validly published.

In 2012, a presumed novel *Mycobacterium* species was repeatedly isolated from respiratory samples of a patient with chronic pulmonary disease at the Nebraska Medical Center and given the strain designation NE-TNMC-100812\(^T\). The ITS region of strain NE-TNMC-100812\(^T\) was sequenced and showed highest sequence similarity to *M. coloregonium* ATCC BAA-1052 (99% similarity and 72% coverage). NE-TNMC-100812\(^T\) was initially assumed to be novel due to low sequence coverage compared to ATCC BAA-1052. We therefore subjected both isolates, ATCC BAA-1052 and NE-TNMC-100812\(^T\), to phenotypic testing, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis and whole genome sequencing to test the hypotheses that (1) the two isolates represent the same species and (2) the isolates belong to a novel species in the genus *Mycobacterium*. Additional analyses of NE-TNMC-100812\(^T\) including fatty acid and mycolic acid profiling and antimicrobial susceptibility testing (AST) were performed to further characterize the novel taxon.

Bacterial cell morphology for the two isolates, NE-TNMC-100812\(^T\) and ATCC BAA-1052, was determined by Ziehl-Neelsen staining methodology. Cells from both isolates were observed to be acid-fast-positive and rod-shaped. Colony morphology, pigment formation and ability to grow at 25, 37 and 42 °C were examined following inoculation on Middlebrook 7H11 media. Colonies grown on 7H11 agar plates were non-pigmented, rough and slightly raised. Growth was observed after 7–10 days at each temperature with optimal growth at 37 °C.

For analysis on the Bruker MALDI-TOF Biotyper system (Bruker Daltonics), freshly grown mycobacteria colonies of NE-TNMC-100812\(^T\) and ATCC BAA-1052 from 7H11 agar plates were isolated and heat-killed in HPLC-grade water, followed by an acetonitrile/formic acid extraction procedure according to the manufacturer’s protocol [5]. The MALDI-TOF target was spotted with 1 µl of extract supernatant and overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid matrix prior to data capture. Each MALDI-TOF spectrum was electronically transformed into a peak list using the Biotyper software v3.1. The peak lists were then compared to a reference database containing peak profiles of 130 species in the genus *Mycobacterium*. Strain designations and NCBI accession numbers are shown next to species names. The phylogenetic tree was reconstructed using a neighbour-joining analysis of full-length 16S rRNA gene sequences. Bootstrap support values of 100 replicate searches are shown at each node. The two major clades representing slowly versus rapidly growing mycobacteria are indicated. Bar, 0.6 single nucleotide polymorphisms.

**Fig. 1.** Phylogenetic analysis of strains NE-TNMC-100812\(^T\) and ATCC BAA-1052 and 24 species in the genus *Mycobacterium*. Strain designations and NCBI accession numbers are shown next to species names. The phylogenetic tree was reconstructed using a neighbour-joining analysis of full-length 16S rRNA gene sequences. Bootstrap support values of 100 replicate searches are shown at each node. The two major clades representing slowly versus rapidly growing mycobacteria are indicated. Bar, 0.6 single nucleotide polymorphisms.
mycobacterial species and a log (score) value between 0.00 and 3.00 was calculated. A log (score) value of ≥2.00 indicates a high probability for organism identification at the species level, a score of 1.7–1.99 suggests low confidence identification and a score <1.7 indicates no significant match to species in the reference database.

NE-TNMC-100812T showed a highest log (score) match of 1.286 while ATCC BAA-1052 had a highest log (score) match of 1.197, suggesting that the novel isolates have distinct MALDI-TOF profiles compared to the 130 mycobacterial species in the reference database. Visual comparisons of MALDI-TOF profiles compared to the 130 mycobacterial species showed similar banding patterns, further suggesting that they belong to the same species (Fig. S1, available in the online Supplementary Material).

For genomic sequencing and phylogenetic analyses, genomic DNA was isolated from NE-TNMC-100812T and ATCC BAA-1052 bacterial pellets according to Kaser et al. [6] except that the phenol/chloroform extraction was replaced with a column-based DNA cleanup (DNeasy from Qiagen). Genomic DNA was sheared using sonication (Covaris) and prepared for whole genome sequencing according to the 400 bp sequencing protocol using the Ion Torrent Personal Genome Machine (Life Technologies).

Sequence reads were filtered for read length and quality and assembled into draft genomes using the gsAssembler algorithm in the Newbler version 2.9 software package (454 Life Sciences). Full-length 16S rRNA (1540 bp) and rpoB (3652 bp) gene sequences were extracted from the genomes and compared between strains NE-TNMC-100812T and ATCC BAA-1052 using custom perl scripts. Then BLAST searches of 16S rRNA, rpoB and hsp65 (957 bp) gene sequences were performed against the National for Biotechnology Information (NCBI) non-redundant nucleotide (nr/nt) database. Pairwise genome comparisons were performed using the average nucleotide identity (ANI) method as a proxy for DNA–DNA hybridization [7]. For phylogenetic comparisons, the 16S rRNA gene sequences were compared among NE-TNMC-100812T and ATCC BAA-1052 and 24 diverse mycobacterial species, and multiple sequence alignment and neighbour-joining analyses were performed using the Seaview software package [8].

Full-length 16S rRNA gene sequences were identical between NE-TNMC-100812T and ATCC BAA-1052. Comparisons of full-length rpoB gene sequences between NE-TNMC-100812T and ATCC BAA-1052 showed 99.7 % similarity (10 single nucleotide polymorphisms in 3265 positions), indicating a low level of genetic divergence between the two isolates. These results support the hypothesis that the two isolates belong to the same species.

Using BLAST analysis against the NCBI nr/nt database, the 16S rRNA gene sequence of strain NE-TNMC-100812T showed highest similarity to the type strain of Mycobacterium simiae (97%), the rpoB gene sequence showed highest similarity to that of Mycobacterium avium (92%) and the hsp65 gene sequence showed highest similarity to that of Mycobacterium sinense (94%). Pairwise genome comparisons between NE-TNMC-100812T and M. simiae ATCC 25275T (NCBI accession number CBMJ000000000) showed an ANI value of 80.03 %, which is well below the accepted cut off of 95–96 % for differentiating two species [9]. The ANI between the genomes of NE-TNMC-100812T and ‘M. avium’ subsp. hominissuis’ strain TH135 (NCBI accession number NZ_AP012555) was 81.5 %. Phylogenetic comparisons of 16S rRNA gene sequences from NE-TNMC-100812T and 24 diverse mycobacterial species (Fig. 1) showed that NE-TNMC-100812T is grouped within the slowly growing subgroup of mycobacteria, which includes M. avium and Mycobacterium tuberculosis, which are characterized as species which are visible in culture after seven or more days of growth. Interestingly, NE-TNMC-100812T is placed as the most basal member of the slowly growing mycobacterial clade, suggesting high genetic relatedness to the most recent common ancestor of that subgroup.

To assess the fatty acid and mycolic acid profiles of NE-TNMC-100812T compared to other mycobacterial species, total lipids from bacterial cells of NE-TNMC-100812T, M. simiae ATCC BAA-1478, M. abscessus ATCC 1977T and M. avium ATCC 700898 were extracted with CHCl3/CH3OH (1:2, v/v) overnight followed by two overnight extractions with CHCl3/CH3OH (2:1, v/v). For TLC analysis and determination of the nature of the alpha branch chain length by GC/MS analysis, mycolic acid methyl esters
originating from the cell wall were prepared from delipidated cells by incubation with 15\% tetrabutylammonium hydroxide (Sigma-Aldrich) overnight at 100 °C followed by methylation with iodomethane (Sigma-Aldrich) for 4 h at room temperature and extraction with dichloromethane. The preparation of mycolic acids for LC/MS analysis followed the procedure described by Bhamidi et al. [10], and LC/MS analysis of mycolic acids followed the procedure described by Grzegorzewicz et al. [11]. GC/MS analyses of fatty acid methyl esters and mycolic acid methyl esters were carried out using a TRACE 1310 gas chromatograph (Thermo Fisher) equipped with a TSQ 8000 Evo Triple Quadrupole in the electron impact mode and scanning from \( m/z \) 70 to \( m/z \) 1000 over 0.8 s. Helium was used as the carrier gas with a flow rate of 1 ml min\(^{-1}\). The samples were run on a ZB-5HT column (15 m × 0.25 mm i.d.) (Zebron). The injector (splitless mode) was set for 300 °C (350 °C for mycolic acid methyl esters). The oven temperature was held at 60 °C for 2 min, programmed at 20 °C min\(^{-1}\) to 375 °C, followed by a 10 min hold. The data analyses were carried out on a Chromeleon data station.

TLC analysis of mycolic acids from NE-TNMC-100812\(^T\) revealed the presence of alpha, keto- and wax ester-mycolates, a profile similar to that found in \( M. avium \) ATCC 700898 (Fig. 2) and \( Mycobacterium xenopi \) [12]. This result was further confirmed by LC/MS analysis. Comparative examination of the total ion chromatograms (Fig. 3) revealed that NE-TNMC-100812\(^T\) was much more closely related to \( M. avium \) ATCC 700898 than to the type strains of \( Mycobacterium abscessus \) or \( M. simiae \) due to the presence of dicarboxylic mycolates, which are formed from the wax ester mycolates during base-catalysed hydrolysis of mycolates in general. NE-TNMC-100812\(^T\) also lacked the \( \alpha'-\)mycolates present in the \( M. abscessus \) and \( M. simiae \) strains. Under the conditions used, the keto-mycolates and the \( \alpha'\)-mycolates co-elute and the diminution of keto-mycolates in NE-TNMC-100812\(^T\) compared to \( M. avium \) is less apparent on the total ion chromatograms than on the TLC chromatograms. However, selective ion traces of the keto-mycolate with 87 carbons showed it was present in NE-TNMC-100812\(^T\) compared to \( M. avium \) ATCC 700898 at a ratio of 0.2/1.

![Image](https://via.placeholder.com/150)

**Fig. 3.** HPLC/MS total ion chromatograms of mycolic acids prepared from strain NE-TNMC-100812\(^T\), \( M. avium \) (ATCC 700898), \( M. abscessus \) (ATCC 19977\(^T\)) and \( M. simiae \) (ATCC BAA-1478).
Finally, AST of NE-TNMC-100812 dominantly 24 carbons in length in NE-TNMC-100812. The results indicated that the alpha-branch is pre-reversed Claisen reaction were identified by their mass spec-GC/MS device, and the released alpha-chains formed by the further subjected to pyrolysis in the injection port of the

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>C16:0</td>
<td>1.0</td>
<td>2.1</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>C18:0</td>
<td>3.4</td>
<td>3.0</td>
<td>28.1</td>
<td>34.4</td>
</tr>
<tr>
<td>C18:1</td>
<td>14.9</td>
<td>28.8</td>
<td>21.5</td>
<td>26.9</td>
</tr>
<tr>
<td>C20:0</td>
<td>7.2</td>
<td>7.4</td>
<td>13.3</td>
<td>9.3</td>
</tr>
<tr>
<td>C20:1</td>
<td>7.2</td>
<td>23.1</td>
<td>9.0</td>
<td>10.5</td>
</tr>
<tr>
<td>10-methyl C18:0</td>
<td>30.0</td>
<td>0.0</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>C22:0</td>
<td>2.1</td>
<td>2.5</td>
<td>5.1</td>
<td>3.4</td>
</tr>
<tr>
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<td>1.4</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.7</td>
<td>1.7</td>
<td>1.9</td>
<td>3.3</td>
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<tr>
<td>C24:1</td>
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<td>0.0</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>C24:2</td>
<td>5.6</td>
<td>3.2</td>
<td>4.9</td>
<td>7.4</td>
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<tr>
<td>C26:0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.3</td>
<td>0.0</td>
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<tr>
<td>C26:1</td>
<td>0.0</td>
<td>0.5</td>
<td>9.7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

To determine the alpha-chain length of mycolates in strain NE-TNMC-100812T, the mycolic acid methyl esters were further subjected to pyrolysis in the injection port of the GC/MS device, and the released alpha-chains formed by the reversed Claisen reaction were identified by their mass spectrum. The results indicated that the alpha-branch is predominantly 24 carbons in length in NE-TNMC-100812T similar to the M. avium and M. abscessus strains, while M. simiae, in contrast, presented alpha-branches predominantly 26 carbons in length, as expected [12].

The fatty acid composition of NE-TNMC-100812T was otherwise typical of that found in other Mycobacterium species with a predominance of C16:0, tuberculostearic acid (10-methyl C18:0) and C18:1. (Table 1).

Finally, AST of NE-TNMC-100812T and the M. avium control strain ATCC 700898 was performed for 10 drugs using the Clinical and Laboratory Standards Institute recommended microdilution method [13]. The AST MIC values of NE-TNMC-100812T differed from the M. avium control strain for six of the 10 drugs (Table 2).

Based on the phylogenetic and phenotypic characterization, Mycobacterium talmoniae sp. nov. is proposed for the two strains, NE-TNMC-100812T and ‘M. coloregonium’ ATCC BAA-1052.

**DESCRIPTION OF MYCOBACTERIUM TALMONIAE SP. NOV.**

*Mycobacterium talmoniae* (tal.mo’ni.aae. N.L. gen. fem. n. talmoniae of Talmon to honour Kathy Talmon, an American microbiologist, for her contributions to the public health laboratory in Nebraska, USA).

Cells are acid-fast-positive using Ziehl-Neelsen staining methodology: AFB-positive rods are short with no cording. Colonies grown on Middlebrook 7H11 agar plates are non-pigmented, rough and slightly raised. Visible growth is observed after 7–10 days on Middlebrook 7H11 agar at 25–42°C. Optimal growth is observed at 37°C. MIC results using a microdilution method show that it is susceptible to rifabutin, moxifloxacin, amikacin, clarithromycin, rifampin and ethambutol, but resistant to clofazimine, linezolid, ciprofloxacin and streptomycin. Has a TLC pattern with alpha-, keto- and wax ester mycolates; this profile is similar to that found in M. avium and other mycobacterial species. The major cellular fatty acids are C16:0, 10 methyl C18:0 and C18:1. Genetically, the organism has unique *Mycobacterium* sequences for the 16S rRNA, rpoB and hsp65 gene targets. Phylogenetic analysis using the 16S rRNA sequence shows that it belongs to the slowly growing mycobacteria clade. Most closely related to M. avium based on the rpoB gene sequence; the ANI between genomes is, however, clearly supportive of the status of independent species.

*Mycobacterium talmoniae* sp. nov. was first observed in patients with pulmonary disease and isolated from human respiratory samples. The type strain, NE-TNMC-100812T (=ATCC BAA-2683T=DSM 46873T), was isolated from human sputum in Nebraska, USA.

**Table 2. MIC results (shown as µg ml⁻¹) for 10 antimicrobials performed by the CLSI recommended microdilution method [13]**

<table>
<thead>
<tr>
<th>Drug</th>
<th>NE-TNMC-100812T</th>
<th>M. avium ATCC 700898</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clofazimine</td>
<td>&gt;0.5</td>
<td>≤0.12</td>
</tr>
<tr>
<td>Rifabutin</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>2.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Linezolid</td>
<td>&gt;64.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>4.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>4.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25</td>
<td>2.0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>5.0</td>
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</table>

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**Conflicts of interest**
The authors declare that they have no conflicts of interest.

**Ethical statement**
Genomic sequencing of NTM isolates derived from patients was approved by the National Jewish Health Internal Review Board under protocol #HS-2674.
References

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