Two novel species of rapidly growing mycobacteria: *Mycobacterium lehmannii* sp. nov. and *Mycobacterium neumannii* sp. nov.

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Abbreviations: *hsp*65, Heat Shock Protein 65; *rpo*B, RNA polymerase β-subunit; dDDH, Digital DNA:DNA Hybridization; ANI, Average Nucleotide Identity; PMG, Proteose peptone-Meat extract-Glycerol; Mb, Megabases (millions of base pairs); MB7H10, Middlebrook 7H10; LJ, Löwenstein Jensen; GYM, Glucose-Yeast extract-Malt extract; TSA, Tryptic Soy Agar; GGDC, Genome-to-Genome Distance Calculator; ML, Maximum-Likelihood; RAxML, Randomized Axelerated Maximum Likelihood; MRE, Maximal-Relative-Error; MP, Maximum-Parsimony; TNT, Tree analysis New Technology; PAUP, Phylogenetic Analysis Using Parsimony; MEGA, Molecular Evolutionary Genetics Analysis; MLSA, Multilocus

Sequence Analysis; A2pm, diaminopimelic acid; PE, PhosphatidylEthanolamine; MIDI, Microbial Identification; GTR, General Time-Reversible; BLAST, Basic Local Alignment Search Tool; MUSCLE, MUltiple Sequence Comparison by Log-Expectation; RAST, Rapid Annotation using Subsystem Technology.

The GenBank accession numbers of strains SN 1900^T and SN 1904^T for 16S rRNA, *hsp*65, *rpo*B gene sequences and genomes are KY933300, KY933786 and KY933788 and KY933299, KY933787 and KY933789, respectively. The genome accession numbers of strains SN 1900^T and SN 1904^T are NKCN00000000 and NKCO00000000, respectively.

Abstract

Two rapidly growing mycobacteria with identical 16S rRNA gene sequences were the subject of a polyphasic taxonomic study. The strains formed a well supported subclade in the mycobacterial 16S rRNA gene tree and were most closely associated with the type strain of *Mycobacterium novocastrense*. Single and multilocus sequence analyses based on *hsp*65, *rpo*B and 16S rRNA gene sequences showed that strains SN 1900^T and SN 1904^T are phylogenetically distinct but share several chemotaxonomic and phenotypic features that are are consistent with their classification in the genus *Mycobacterium*. The two strains were distinguished by their different fatty acid and mycolic acid profiles and by a combination of phenotypic features. Digital DNA:DNA hybridization (dDDH) and average nucleotide identity (ANI) values for strains SN 1900^T and SN 1904^T were 61 % and 94.7%, respectively; in tum, the corresponding dDDH and ANI values with *M. novovastrense* DSM 44203^T were 41.4% and 42.8% and 89.3 % and 89.5 %. These results show that strains SN1900^T and SN 1904^T form new centres of taxonomic variation within the genus *Mycobacterium*. Consequently, strains SN 1900^T (40^T = CECT 8763 ^{T} = DSM 43219^T) and SN 1904^T (2409^T = CECT 8766 ^{T} = DSM 43532^T) are considered to represent novel species for which the names *Mycobacterium lehmannii* sp. nov. and *Mycobacterium neumannii* sp. nov. are proposed. A strain designed as "Mycobacterium acapulsensis" was shown to be a *bona fid*e member of the putative novel species, *M. lehmannii.*

Mycobacterium [1], the type genus of the family *Mycobacteriaceae* [2], accommodates diverse pathogenic and non-tuberculous mycobacteria [3, 4] that are common in the environment and which can be opportunistic pathogens. At the time of writing, the genus *Mycobacterium* encompasses more than 165 species with validly published names [5]. These species can be classified into two groups, slowly growing mycobacteria that form viable colonies from highly diluted inocula following incubation for seven or more days at optimal temperature whereas colonies of their rapidly growing counterparts are seen within seven days or less under comparable conditions [6]. Combinations of genotypic and phenotypic criteria, including mycolic acid type, are now used to describe new mycobacterial species and have been shown to be especially useful in distinghuishing between closely related species such as those previously grouped together within the *Mycobacterium abscessus* and *Mycobacterium avium* complexes [7-9]. Such improvements in mycobacterial systematics are needed to detect the

causal agents of mycobacterial infections and to establish the primary reservoirs of individual mycobacterial species [4, 10-12].

In the present study, two rapidly growing mycobacteria of unknown provenance were the subject of a polyphasic study designed to establish their taxonomic status within the genus *Mycobacterium*. The resultant data showed that the two strains, designated SN1900^T and SN1904^T, belong to two different species for which the names *Mycobacterium lehmannii* sp. nov. and *Mycobacterium neumannii* sp. nov. are proposed.

Strains SN 1900^T (CECT 8763^T) and SN 1904^T (CECT 8766^T) were originally deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) by Dr. Ivan Tárnok in the early 1990's as *Mycobacterium flavescens* [13] and were given the accession numbers DSM 43532^T and DSM 43219^T, respectively. Strain SN 1900^T was isolated by the late John Grange at the Institute of Pathology, London but the origin of strain SN 1904^T remains unknown. Preliminary studies based on 16S rRNA gene sequence data showed that they are closely related to the type strain of *Mycobacterium novocastrense* [14]. All three of these strains were maintained on proteose peptone-meat extract-glycerol agar (PMG; DSM 250 medium) and as suspensions in 30%, v/v glycerol at -80°C.

The colonial and pigmentation properties of the isolates were determined on Löwenstein - Jensen medium (LJ; [15]), Middlebrook 7H10 agar (MB7H10; [16]), proteose peptone-yeast extract-glycerol agar, glucose-yeast extract-malt extract agar (GYM; DSM medium 65) and tryptic soy agar (TSA; [17]) after incubation for 14 days at 10°C, 25°C, 28°C, 37°C and 45°C under light and dark conditions. The strains were examined for acid-alcohol-fastness using the Zielhl-Neelsen method [18] and shown to be acid alcohol-fast. They produced yellow orange colonies under both light and dark conditions on the GYM, LJ, Middlebrook 7H10, PMG and TSA plates after 5 days at 37°C. Optimal growth of both strains was detected at 37°C on GYM, MB7H10 and PMG agar after 5 days. Both strains were able to grow under anaerobic conditions at 37°C on GYM agar using an anaerobic bag system (Sigma-Aldrich 68061**)** but did not grow on any of the media at 10°C, 25°C or 45°C.

Genomic DNA was extracted from strains SN 1900^T and SN 1904^T using the protocol of Amaro *et al.* [19]. Genome sequencing was performed on an Illumina MiSeq instrument as previously described [20] and the genomes assembled into contigs using SPAdes 3.9.0 with a kmer length of 127 [21]. Complete 16S rRNA gene sequences of the strains were extracted from the draft genome sequences (accession numbers NKCN00000000 and NKCO00000000) and deposited

in GenBank (accession numbers KY933299 and KY933300). Corresponding sequences of the type strains of closely related *Mycobacterium* species were retrieved using the EzBioCloud server [22] and pairwise sequence similarities calculated with the genome to genome distance calculator (GGDC) web server [23, 24]. Phylogenetic analyses were carried out using the GGDC web server and the DSMZ phylogenomic pipeline [25] adapted for single genes. Multiple sequence alignments were generated using MUSCLE software [26]. A maximum-likelihood (ML) tree was inferred from the alignment with RAxML [27] using rapid bootstrapping together with the auto MRE criterion [28]. In tum, a maximum-parsimony (MP) tree was inferred from the alignment with the TNT program [29] using 1000 bootstraps together with tree bisection and reconnection branch swapping and ten random sequence replicates. The sequences were checked for compositional bias using the X^2 test implemented in PAUP* [30]. A multilocus sequence analysis based on partial sequences of two housekeeping genes, namely *hsp*65 (heat shock protein) and *rpo*B (RNA polymerase β-subunit) was performed including corresponding 16S rRNA gene sequence data; these housekeeping genes are well known for their effectiveness in clarifying relationships between closely related taxa in the genus *Mycobacterium* [10, 31]. The maximum-likelihood method together with the Kimura 2-parameter model [32] and MEGA version 7 software were used for the construction of a phylogenetic tree from concatenated sequences of the genes mentioned above.

BLAST analysis of the complete 16S rRNA gene sequences of strains SN 1900^T and SN 1904^T showed 98.3 % gene sequence similarity to both *Mycobacterium moriokaense* DSM 44221^T [33] and *M. novocastrense* DSM 44203^T [14]. The corresponding pairwise similarities between the test strains and the type strains of *M. moriokaense* and *M. novocastrense* were 98.3% and 98.5%, respectively. These pairwise similarities are in line with the topology of the 16S rRNA gene tree in which strains SN 1900^T and SN 1904^T formed a distinct well-supported subclade close to *M. novocastrense* DSM 44203^T while the *M. moriokaense* strain was assigned to a subclade together with *Mycobacterium celeriflavum* [34] and *Mycobacterium thermoresistibile* [35] (Fig. 1). The 16S rRNA gene sequence similarity between strains SN1900^T and SN 1904^T was 100% but decreased to 97.5%, 98.0% and 98.4% against *Mycobacterium doricum* [36], *Mycobacterium monacense* [37] and *Mycobacterium vaccae* [38], respectively, which were recovered in an associated subclade. However, in the MLSA tree that was based on concatenated sequences of the *hsp*65, *rpo*B and 16S rRNA genes (Fig. 2), strains SN 1900^T and SN 1904^T formed a well supported subclade that was sharply separated from an adjacent taxon that encompassed the type strains of *Mycobacterium brumae* [39], *Mycobacterium*

flavescens [13], *Mycobacterium holsaticum* [40], *M. moriokaense* [33], *M. novocastrense* [14], *Mycobacterium setense* [41] and *Mycobacterium vaccae* [38]. The genetic distances derived from the multilocus sequence analyses between the test strains and their phylogenetic neighbours were in very good agreement with the 16S rRNA gene data. Genetic distances of 0.107 and 0.106 were recorded between strains SN 1900^T and SN 1904^T and *M. novocastrense* DSM 44203^T, values that rose to 0.117 and 0.118 against the *M. moriokaense* strain. It can be concluded from all of these data that isolates SN 1900^T and SN 1904^T form a well supported subclade within the evolutionary radiation occupied by rapidly growing mycobacteria, a relationship that is underlined by the absence of 18 nucleotides at site 480 (*Escherichia coli* 16S rRNA gene position) in hypervariable region B of the 16S rRNA gene, this deletion is known to be a characteristic feature of fast growing mycobacteria [42].

The genome sizes of strains SN1900^T and SN 1904^T were found to be ~5.5 Mb and ~5.4 Mb with an average *in silico* G+C content of 66.7 mol% and 66.8 mol%, respectively. These genomes are smaller than the ~6.2 Mb genome of *M. novocastrense* DSM 44203^T [43]. The assembly size, coverage and contig numbers for strains SN 1900^T and SN1904^T were 5.5Mb and 5.4MB; 47X and 81X, 76 and 81, respectively. The genomes of strains SN 1900^T and SN1904^T were annotated using the RAST pipeline [44, 45] and shown to have 5,360 (51 RNAs) and 5,154 (52 RNAs) coding sequences, respectively. The average nucleotide identity (ANI) between the genomes of the test strains and the type strain of *M. novocastrense*, their close phylogenetic neighbour, was calculated using the blastANI algorithm [46]. Digital DNA:DNA hybridization (dDDH) values between isolates SN 1900^T, SN 1904^T and the *M. novocastrense* strain were estimated using the GGDC based on formula 2 of Meier-Kolthoff *et al.* [23] which is available on the DSMZ website (http://ggdc.dsmz.de/).
The dDDH relatedness between strains SN 1900^T and SN 1904^T was 61.0%, a value well below the threshold of 70% for assigning bacterial strains to the same species [47]. The corresponding ANI relatedness value between these strains was 94.7%, a cut off well below the threshold of 95-96 % used for prokaryotic species delineation [46, 48, 49]. In tum, the dDDH values between strains SN 1900^T and SN 1904^T and the type strain of *M. novocastrense* were 41.4% and 42.8 %, respectively, and the corresponding ANI values 89.3 % and 89.5 %.

Biomass for the chemotaxonomic analyses, apart from the fatty acid studies, was harvested from shake flasks (200 revolutions per minute) of PMG broth (DSM medium 250) after 7 days at 37°C, washed three time in sodium chloride solution (0.9%, w/v) and freeze dried. The same

procedure was used to obtain biomass for the fatty acid analyses but in this case cells were grown in Middelbrook 7H10 broth. Standard thin-layer chromatographic procedures were used to establish the chemotaxonomic profiles of strains SN 1900^T and SN 1904^T. To this end, they were examined for isomers of diaminopimelic acid (A_2pm) [50]; predomimant isoprenologues [51, 52]; whole organism sugars [53]; and for polar lipids using the procedure of Minnikin *et al.* [54]. The isolates were found to contain *meso*-A_2pm, MK-9(H_2) as the predominant menaquinone (>95%), whole cell sugar profiles containing arabinose, galactose, glucose and ribose, and to have polar lipid patterns consisting of diphosphatidylglycerol, glycophospholipid, phosphatidylethanolamine (PE), phosphatidylinositol, two aminolipids, a glycolipid and an unidentified lipid; the *M. novocastrense* DSM 44203^T lacked PE and galactose.

Cellular fatty acids were extracted from the isolates and from *M. novocastrense* DSM 44203^T and methylated according to Miller [55], as modified by Kuykendall *et al*. [56], then analysed by gas chromatography (Agilent 6890N instrument). The resultant peaks were integrated and the fatty acids identified using the standard Microbial Identification (MIDI) system, version 4.5 and the myco5 database [57]. Mycolic acid methyl esters prepared from these strains after Minnikin and Goodfellow [58] were separated and identified, as described by Teramoto *et al.* [59]. All three strains contained similar mixtures of saturated, unsaturated and 10-methyloctadecanoic (tubercolostearic) fatty acids though quantitative differences were found with respect to both major and minor components (Table 1). The major fatty acid (>25%) detected in isolate SN 1904^T was $C_{16:0}$ while isolate SN 1900^T and *M. novocastrense* DSM 44203^T contained a predominant proportion of fatty acids summed in feature 3 (Table 1). Strains SN 1900^T and SN 1904^T contained dicarboxy mycolic acids with 61-64 carbon atoms but *keto*-mycolates with 78-83 carbon atoms were found only in isolate SN 1904^T (Fig. S1).

The two test strains and *M. novocastrense* DSM 44203^T were examined for a broad range of standard phenotypic procedures using media and methods described by Nouioui *et al.* [60]; these tests were carried out in triplicate using freshly prepared inocula (OD_{600} 0.3-0.6) harvested from the mid-logarithmic growth phase using PMG agar as the cultivation medium and incubation at 37°C. Biochemical tests known to be of value in mycobacterial systematics were performed on strains SN1900^T and SN1904^T, namely detection of arylsulfatase after 3 and 20 days [61], catalase [62], heat stable catalase [63], niacin accumulation [64] and nitrate reductase [65]. The strains were also examined for the reduction of potassium tellurite [64, 66], degradation of Tween 80 [67] and urea hydrolysis [62]. Additional enzymatic tests were determined using API ZYM kits, as instructed by the manufacturer (Biomérieux, France). The ability of the strains to oxidise carbon and nitrogen sources and to show resistance to inhibitory

compounds were evaluated using GENIII microplates in an Omnilog device (Biolog Inc., Hayward, USA), as described by Nouioui *et al.* [60].The duplicated sets of exported data derived from the microplates were analysed using opm package version 1.3.36 [68, 69].

Identical results were obtained for all of the duplicated and triplicated phenotypic tests. Several phenotypic features were weighted to distinguish isolates SN 1900^T and SN 1904^T from one another and from *M. novocastrense* DSM 44203^T (Table 2). Strain SN 1904^T can be separated from strain SN 1900^T by its ability to degrade Tween 80, to oxidise dextrin, glucuronamide, N-acetyl-D-glucosamine and γ-amino-*n*-butyric acid and by its resistance to inhibitory compounds such as guanidine hydrochloride, tetrazolium blue, tetrazolium violet and vancomycin. In contrast, only isolate SN 1900^T produced α-and β-glucosidases and nitrate reductase, utilised D-cellobiose, D-fucose, D-glucuronic acid, glycine-proline and D- and L-malic acid, and grew in presence of Tween 40. The two test strains can be distinguished from the *M. novocastrense* strain by several phenotypic features, such as their ability to oxidise D-arabitol, α-hydroxy-butyric acid, D-fructose, D-mannitol, D-serine and D-sorbitol (Table 1). In contrast, *M. novocastrense* DSM 44203^T, unlike the isolates, oxidises D-glucose-6-phosphate, D-galacturonic acid and D-turanose. Strains SN1900^T and SN 1904^T were found to produce catalase, heat stable (68°C) catalase and urease; accumulated niacin; and were not inhibited by potassium tellurite.

It can be concluded from the wealth of phylogenetic, dDDH, chemotaxonomic and phenotypic data that strains SN 1900^T and SN 1904^T can be distinghuished from one another and from *M. novocastrense* DSM 44203^T their close phylogenetic neighbour. Consequently, strains SN 1900^T and SN 1904^T are considered to represent two novel species within the genus *Mycobacterium*, namely as *Mycobacterium lehmannii* sp. nov. and *Mycobacterium neumannii* sp. nov., respectively.

A draft genome sequence is available for "Mycobacterium acapulcensis*"* CSURP1424, a strain isolated from sputum of a patient with pulmonary lesions during a compaign against tuberculosis [70]. However, this binomial name has not been validly published and hence has no standing in nomemclature [71]. Strain CSURP1424 and SN 1900^T share a dDDH value of 81.3% and an ANI value of 98.3%, results well above the thresholds for assigning strains to the same species [46-49]. Consequently, strain CSURP1424 is a *bona fide* member of the putative novel species, *M. lehmannnii.*

Description of *Mycobacterium lehmannii* sp. nov.

Mycobacterium lehmannii (leh.man'ni.i N.L. gen. n. *lehmannii*, named after Karl Bernhard Lehmann, German hygienist who together with Rudolf Otto Neumann proposed the genus *Mycobacterium*)

Gram-stain positive, acid-alcohol fast, facultatively anaerobic, non-motile, fast growing organism which produces yellow-orange coloured colonies on Middelbrook 7H10, proteose peptone-meat extract-glycerol, glucose-yeast extract-malt extract and tryptic soy agar plates within 5 days at 37°C under dark and light conditions. Grows between 28°C and 37°C, optimally ~ 37°C, and at pH7 and in the presence of 4% w/v sodium chloride. Produces arylsulfatase within 3 and 20 days and reduces nitrate. Additional phenotypic tests are cited in the text and in Table 2. *Meso*-diaminopimelic acid, arabinose, galactose, glucose and ribose are present in whole organism hydrolysates and the predominant menaquinone is MK-9(H_2). The polar lipid profile is composed of diphosphatidylglycerol, glycophospholipid, phosphatidyethanolamine, phosphatidylinositol, two aminolipids and an unidentified lipid and the major fatty acid (>20%) is summed feature 3. Contains dicarboxy mycolic acids with 61-64 carbon atoms. The *in silico* DNA G+C content is 66.7%. The type strain is SN 1900^T (40^T = CECT 8763^T = DSM 43219^T).

Description of *Mycobacterium neumannii* sp. nov.

Mycobacterium neumannii (ne.u.man'ni.i N.L. gen. n. *neumannii*, named after Rudolf Otto Neumann, German microbiologists who together with Karl Bernhard Lehmann proposed the genus *Mycobacterium*)

Gram-stain positive, acid-alcohol fast, facultatively anaerobic, non-motile, fast growing organism which produces yellow-orange coloured colonies on Middelbrook 7H10, proteose peptone-meat extract-glycerol, glucose-yeast extract-malt extract and tryptic soy agar plates within 5 days at 37°C under dark and light conditions. Grows between 28°C and 37°C, optimally ~37°C, at pH7 and in the presence of up to 8% w/v sodium chloride. Produces arylsulfatase after 3 and 20 days but not nitrate reductase. Additional phenotypic tests are cited in the text and in Table 2. *Meso*-diaminopimelic acid, arabinose, galactose, glucose and ribose are present in whole organism hydrolysates and the predominant menaquinone is MK-9(H_2). The polar lipid profile consists of diphosphatidylglycerol, glycophospholipid, phosphatidyethanolamine, phosphatidylinositol, two aminolipids, glycolipid and an unidentified lipid and the major fatty acid (>20%) is $C_{16:0}$. Contains dicarboxy mycolic acids

with 61-64 carbon atoms and *keto*-mycolates with 78-83 carbon atoms. The *in silico* DNA G+C content of strain SN 1904^T is 66.8%. The type strain is SN 1904^T (2409^T = CECT 8766 ^{T} = DSM 43532^T).

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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456 **Table 1.** Fatty acid profiles of strains SN 1900^T, SN 1904^T and the type strain of *M.*
457 *novocastrense*
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Fatty acids	**SN 1900^T**	**SN 1904^T**	***M. novocastrense* DSM 44203^T**
$C_{16:0}$	17.4	25.2	15.4
$C_{16:1}$ ω6c	7.5	8.7	6.7
$C_{16:1}$ ω9c	-	-	1.3
$C_{18:0}$	2.9	4.7	1.4
$C_{18:1}$ ω9c	8.9	8.9	11.1
$C_{18:2}$ ω6,9c	3.3	1.4	1.2
C_{10Me}-$C_{18:0}$	6.1	5.6	4.7
$C_{14:0}$	2.2	3.7	2.0
Summed feature 2*	18.5	15.4	24.0
Summed feature 3**	31.3	22.0	29.9

459 * $C_{17:1}$ ω7c / $C_{17:1}$ ω6c ** $C_{20:0}$ ALC 18.838 ECL /20 alcohol / $C_{19:0}$ Cycloprop ω10c /
460 $C_{19:0}$ Cycloprop ω8c.
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471 **Table 2**. Phenotypic features that distinguish strains SN 1904^T and SN 1900^T from one another
472 and from the type strain of *M. novocastrense*

	SN 1900^T	**SN 1904^T**	***M. novocastrense* DSM 44203^T**
Biochemical tests:			
α- and β-Glucosidase, nitrate reductase	+	-	-
Catalase, catalase heat stable 68°C, urease, niacin accumulation	+	+	-
Utilisation of sugars :			
D-Arabitol, D-fructose, D-mannitol, D-sorbitol,	+	+	-
D-Cellobiose, D-fucose	+	-	+
Dextrin	-	+	-
D-Glucose-6-phosphate, D-turanose	-	-	+
N-acetyl-D-Glucosamine	-	+	+
Utilisation of amino acids :			
Glucuronamide	-	+	-
Glycine-proline	+	-	-
D-Serine	+	+	-
Utilisation of organic acids:			
γ-amino-*n*-Butyric acid	-	+	-
α-hydroxy-Butyric acid	+	+	-
D-Galacturonic acid	-	-	+
D-Glucuronic acid	+	-	+
D-and L-Malic acid	+	-	-
Resistance to:			
Sodium bromate	-	+	-
Sodium chloride (4% w/v), sodium formate, sodium lactate (1%), potassium tellurite	+	+	-
Sodium chloride (8% w/v)	-	+	-
Guanidine hydrochloride, tetrazolium blue, tetrazolium violet, vancomycin	-	+	-
Degradation tests:			
Tween 80	-	+	-
Tween 40	+	-	-

473 + Positive reaction; - negative reaction.

All of the strains produced esterase (C4) and alkaline phosphatase, utilised acetic acid, acetoacetic acid, butyric acid, β-hydroxy-butyric acid, α-*keto*-glutaric acid, citric acid, D-fructose-6-phosphate, D-gluconic acid, D-glucose, D-mannose, D-salicin, D-trehalose, glycerol, L-glutamic acid, methyl pyruvate and propionic acid and were not inhibited by aztreonam, lithium chloride, nalidixic acid, rifamycin sv or potassium tellurite. In contrast, none of the strains utilised D or L-aspartic acid, L-alanine, L-arginine, α-D-lactose, α-*keto*-butyric acid, β-gentiobiose, β-methyl-D-glucoside, bromo-succinic acid, , D-galactose, D-lactic acid methyl ester, D-maltose, D-melibiose, D-raffinose, D-saccharic acid, gelatin, inosine, L-fucose, L-galactonic acid-γ-lactone, L-histidine, L-lactic acid, L-pyroglutamic acid, L-rhamnose, L-serine, mucic acid, *myo*-inositol, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-neuraminic acid, 3-*O*-methyl-D-glucose, pectin, *p*-hydroxy-phenylacetic acid, quinic acid, stachyose or sucrose and were inhibited by fusidic acid, lincomycin, minocycline, niaproof and troleandomycin.

Figure legends

Fig. 1. Maximum-likelihood phylogenetic tree based on almost complete16S rRNA gene sequences inferred using the GTR+GAMMA model and rooted by midpoint-rooting. The numbers above the branches are bootstrap support values greater than 60% for ML (left) and MP (right).

Fig. 2. Maximum-likelihood MLST phylogenetic tree constructed from 2332 nucleotide concatenated sequences of 16S rRNA, *hsp*65 and *rpo*B genes. Bootstrap values above 50% are displayed.

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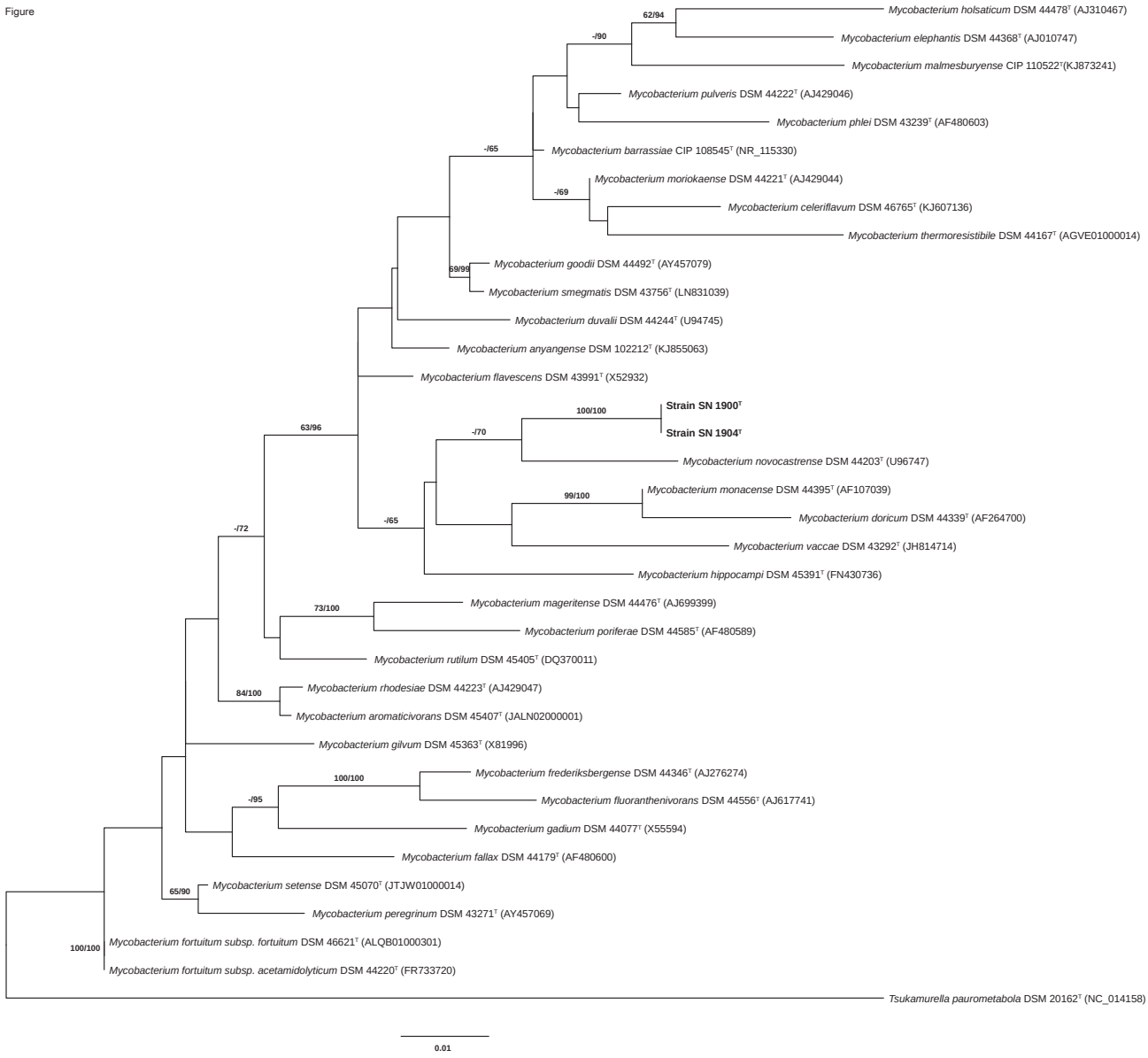
Figure


62/94
-/90
-/65
-/69
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-/70
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84/100
100/100
-/95
65/90
100/100
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Mycobacterium malmesburyense CIP 110522T(KJ873241)
Mycobacterium pulveris DSM 44222T (AJ429046)
Mycobacterium phlei DSM 43239T (AF480603)
Mycobacterium barrassiae CIP 108545T (NR_115330)
Mycobacterium moriokaense DSM 44221T (AJ429044)
Mycobacterium celeriflavum DSM 46765T (KJ607136)
Mycobacterium thermoresistibile DSM 44167T (AGVE01000014)
Mycobacterium goodii DSM 44492T (AY457079)
Mycobacterium smegmatis DSM 43756T (LN831039)
Mycobacterium duvalii DSM 44244T (U94745)
Mycobacterium anyangense DSM 102212T (KJ855063)
Mycobacterium flavescens DSM 43991T (X52932)
Strain SN 1900T
Strain SN 1904T
Mycobacterium novocastrense DSM 44203T (U96747)
Mycobacterium monacense DSM 44395T (AF107039)
Mycobacterium doricum DSM 44339T (AF264700)
Mycobacterium vaccae DSM 43292T (JH814714)
Mycobacterium hippocampi DSM 45391T (FN430736)
Mycobacterium mageritense DSM 44476T (AJ699399)
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Mycobacterium frederiksbergense DSM 44346T (AJ276274)
Mycobacterium fluoranthenivorans DSM 44556T (AJ617741)
Mycobacterium gadium DSM 44077T (X55594)
Mycobacterium fallax DSM 44179T (AF480600)
Mycobacterium setense DSM 45070T (JTJW01000014)
Mycobacterium peregrinum DSM 43271T (AY457069)
Mycobacterium fortuitum subsp. fortuitum DSM 46621T (ALQB01000301)
Mycobacterium fortuitum subsp. acetamidolyticum DSM 44220T (FR733720)
Tsukamurella paurometabola DSM 20162T (NC_014158)

Figure

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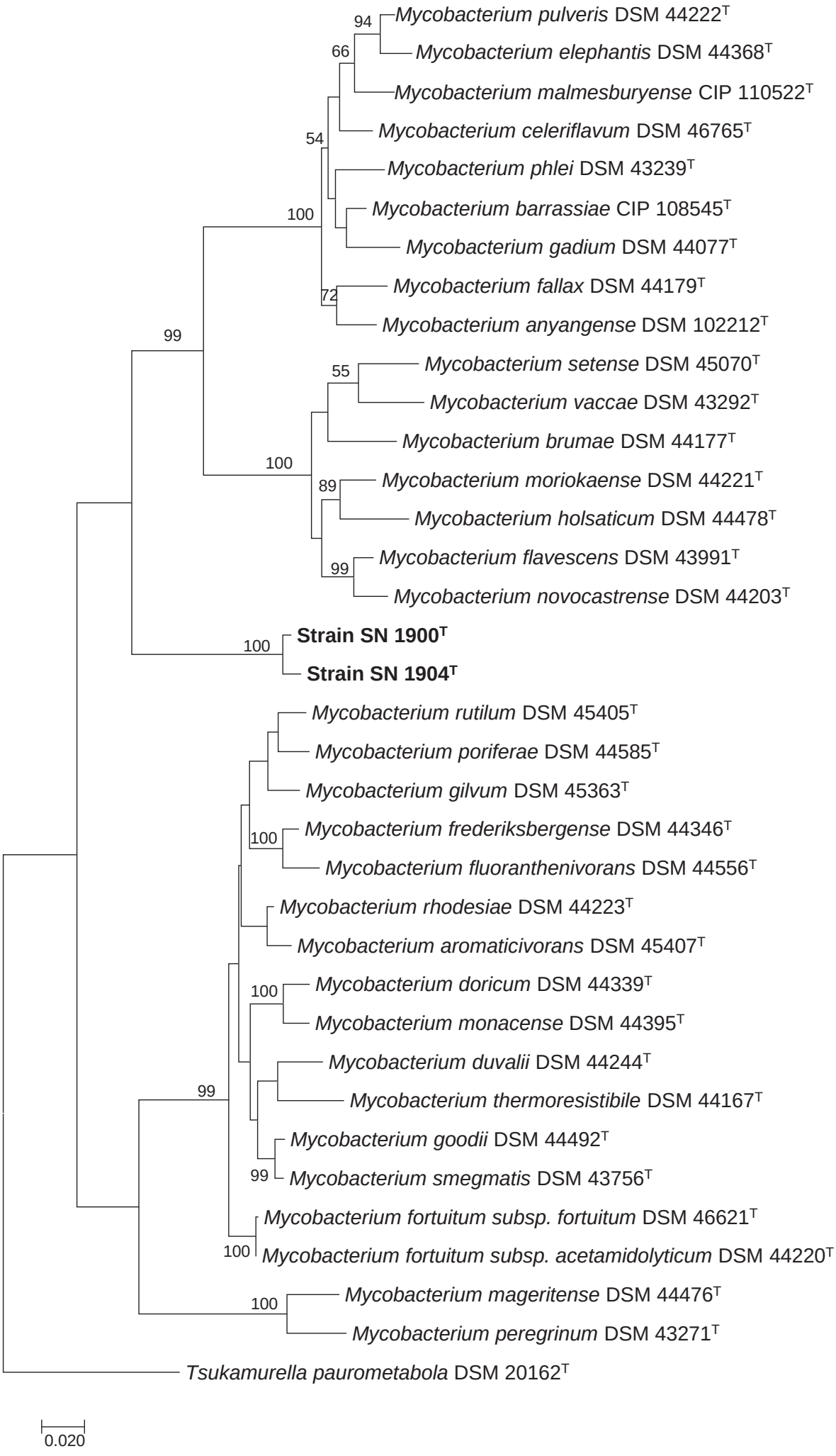

Supplementary data

Two novel species of rapidly growing mycobacteria: *Mycobacterium lehmannii* sp. nov. and *Mycobacterium neumannii* sp. nov.

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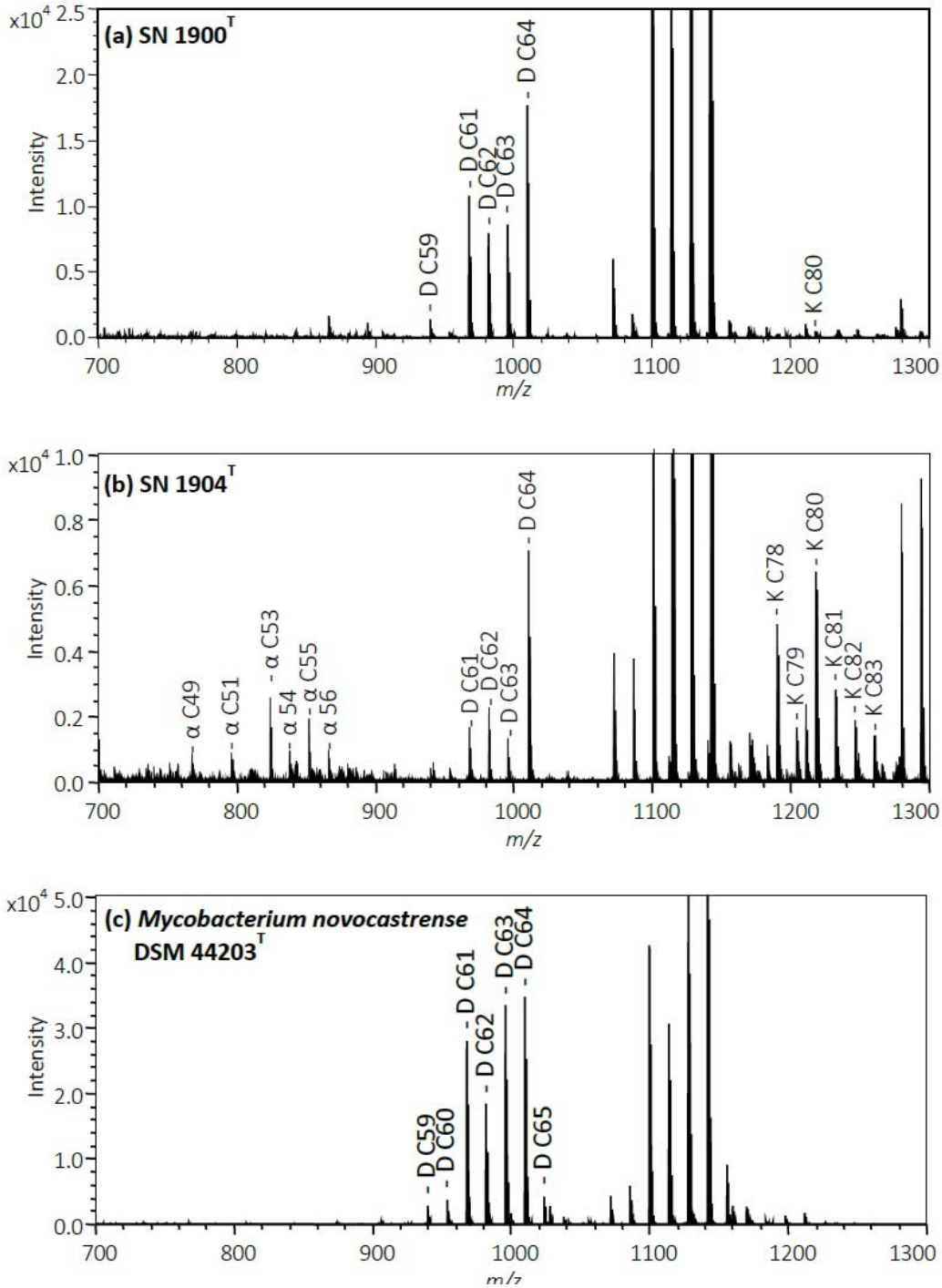

Fig. S1. Mycolic acid profiles of strains SN1900 ^{T} (a), SN1904^{T} (b) and *M. novocastrense* DSM 44203^{T} (c). D = dicarboxy-mycolic acid; α = *α*-mycolic acid, K= *keto*-mycolic acid.