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

3
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23
24 **Section:** Actinobacteria

25
26 **Keywords:** Actinobacteria, polyphasic taxonomy, draft-genome sequencing

27 **Abbreviations:** *hsp65*, Heat Shock Protein 65; *rpoB*, RNA polymerase β -subunit; dDDH,
28 Digital DNA:DNA Hybridization; ANI, Average Nucleotide Identity; PMG, Proteose peptone-
29 Meat extract-Glycerol; Mb, Megabases (millions of base pairs); MB7H10, Middlebrook 7H10;
30 LJ, Löwenstein Jensen; GYM, Glucose-Yeast extract-Malt extract; TSA, Tryptic Soy Agar;
31 GGDC, Genome-to-Genome Distance Calculator; ML, Maximum-Likelihood; RAxML,
32 Randomized Axelerated Maximum Likelihood; MRE, Maximal-Relative-Error; MP,
33 Maximum-Parsimony; TNT, Tree analysis New Technology; PAUP, Phylogenetic Analysis
34 Using Parsimony; MEGA, Molecular Evolutionary Genetics Analysis; MLSA, Multilocus

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

39

40 The GenBank accession numbers of strains SN 1900^T and SN 1904^T for 16S rRNA, *hsp65*,
41 *rpoB* gene sequences and genomes are KY933300, KY933786 and KY933788 and KY933299,
42 KY933787 and KY933789, respectively. The genome accession numbers of strains SN 1900^T
43 and SN 1904^T are NKCN00000000 and NKCO00000000, respectively.

44

45

46 **Abstract**

47

48 Two rapidly growing mycobacteria with identical 16S rRNA gene sequences were the subject
49 of a polyphasic taxonomic study. The strains formed a well supported subclade in the
50 mycobacterial 16S rRNA gene tree and were most closely associated with the type strain of
51 *Mycobacterium novocastrense*. Single and multilocus sequence analyses based on *hsp65*, *rpoB*
52 and 16S rRNA gene sequences showed that strains SN 1900^T and SN 1904^T are
53 phylogenetically distinct but share several chemotaxonomic and phenotypic features that are
54 are consistent with their classification in the genus *Mycobacterium*. The two strains were
55 distinguished by their different fatty acid and mycolic acid profiles and by a combination of
56 phenotypic features. Digital DNA:DNA hybridization (dDDH) and average nucleotide identity
57 (ANI) values for strains SN 1900^T and SN 1904^T were 61 % and 94.7%, respectively; in tum,
58 the corresponding dDDH and ANI values with *M. novovastrense* DSM 44203^T were 41.4% and
59 42.8% and 89.3 % and 89.5 %. These results show that strains SN1900^T and SN 1904^T form
60 new centres of taxonomic variation within the genus *Mycobacterium*. Consequently, strains SN
61 1900^T (40^T = CECT 8763^T = DSM 43219^T) and SN 1904^T (2409^T = CECT 8766^T = DSM
62 43532^T) are considered to represent novel species for which the names *Mycobacterium*
63 *lehmannii* sp. nov. and *Mycobacterium neumannii* sp. nov. are proposed. A strain designed as
64 “*Mycobacterium acapulcensis*” was shown to be a *bona fide* member of the putative novel
65 species, *M. lehmannii*.

66

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

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

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

86 Strains SN 1900^T (CECT 8763^T) and SN 1904^T (CECT 8766^T) were originally deposited in the
87 German Collection of Microorganisms and Cell Cultures (DSMZ) by Dr. Ivan Tárnok in the
88 early 1990's as *Mycobacterium flavescens* [13] and were given the accession numbers DSM
89 43532^T and DSM 43219^T, respectively. Strain SN 1900^T was isolated by the late John Grange
90 at the Institute of Pathology, London but the origin of strain SN 1904^T remains unknown.
91 Preliminary studies based on 16S rRNA gene sequence data showed that they are closely related
92 to the type strain of *Mycobacterium novocastrense* [14]. All three of these strains were
93 maintained on proteose peptone-meat extract-glycerol agar (PMG; DSM 250 medium) and as
94 suspensions in 30%, v/v glycerol at -80°C.

95
96 The colonial and pigmentation properties of the isolates were determined on Löwenstein -
97 Jensen medium (LJ; [15]), Middlebrook 7H10 agar (MB7H10; [16]), proteose peptone-yeast
98 extract-glycerol agar, glucose-yeast extract-malt extract agar (GYM; DSM medium 65) and
99 tryptic soy agar (TSA; [17]) after incubation for 14 days at 10°C, 25°C, 28°C, 37°C and 45°C
100 under light and dark conditions. The strains were examined for acid-alcohol-fastness using the
101 Zielhl-Neelsen method [18] and shown to be acid alcohol-fast. They produced yellow orange
102 colonies under both light and dark conditions on the GYM, LJ, Middlebrook 7H10, PMG and
103 TSA plates after 5 days at 37°C. Optimal growth of both strains was detected at 37°C on GYM,
104 MB7H10 and PMG agar after 5 days. Both strains were able to grow under anaerobic conditions
105 at 37°C on GYM agar using an anaerobic bag system (Sigma-Aldrich 68061) but did not grow
106 on any of the media at 10°C, 25°C or 45°C.

107 Genomic DNA was extracted from strains SN 1900^T and SN 1904^T using the protocol of Amaro
108 *et al.* [19]. Genome sequencing was performed on an Illumina MiSeq instrument as previously
109 described [20] and the genomes assembled into contigs using SPAdes 3.9.0 with a kmer length
110 of 127 [21]. Complete 16S rRNA gene sequences of the strains were extracted from the draft
111 genome sequences (accession numbers NKCN000000000 and NKCO000000000) and deposited

112 in GenBank (accession numbers KY933299 and KY933300). Corresponding sequences of the
113 type strains of closely related *Mycobacterium* species were retrieved using the EzBioCloud
114 server [22] and pairwise sequence similarities calculated with the genome to genome distance
115 calculator (GGDC) web server [23, 24]. Phylogenetic analyses were carried out using the
116 GGDC web server and the DSMZ phylogenomic pipeline [25] adapted for single genes.
117 Multiple sequence alignments were generated using MUSCLE software [26]. A maximum-
118 likelihood (ML) tree was inferred from the alignment with RAxML [27] using rapid
119 bootstrapping together with the auto MRE criterion [28]. In tum, a maximum-parsimony (MP)
120 tree was inferred from the alignment with the TNT program [29] using 1000 bootstraps together
121 with tree bisection and reconnection branch swapping and ten random sequence replicates. The
122 sequences were checked for compositional bias using the X^2 test implemented in PAUP* [30].
123 A multilocus sequence analysis based on partial sequences of two housekeeping genes, namely
124 *hsp65* (heat shock protein) and *rpoB* (RNA polymerase β -subunit) was performed including
125 corresponding 16S rRNA gene sequence data; these housekeeping genes are well known for
126 their effectiveness in clarifying relationships between closely related taxa in the genus
127 *Mycobacterium* [10, 31]. The maximum-likelihood method together with the Kimura 2-
128 parameter model [32] and MEGA version 7 software were used for the construction of a
129 phylogenetic tree from concatenated sequences of the genes mentioned above.

130 BLAST analysis of the complete 16S rRNA gene sequences of strains SN 1900^T and SN 1904^T
131 showed 98.3 % gene sequence similarity to both *Mycobacterium moriokaense* DSM 44221^T
132 [33] and *M. novocastrense* DSM 44203^T [14]. The corresponding pairwise similarities between
133 the test strains and the type strains of *M. moriokaense* and *M. novocastrense* were 98.3% and
134 98.5%, respectively. These pairwise similarities are in line with the topology of the 16S rRNA
135 gene tree in which strains SN 1900^T and SN 1904^T formed a distinct well-supported subclade
136 close to *M. novocastrense* DSM 44203^T while the *M. moriokaense* strain was assigned to a
137 subclade together with *Mycobacterium celeriflavum* [34] and *Mycobacterium thermoresistibile*
138 [35] (Fig. 1). The 16S rRNA gene sequence similarity between strains SN1900^T and SN 1904^T
139 was 100% but decreased to 97.5%, 98.0% and 98.4% against *Mycobacterium doricum* [36],
140 *Mycobacterium monacense* [37] and *Mycobacterium vaccae* [38], respectively, which were
141 recovered in an associated subclade. However, in the MLSA tree that was based on
142 concatenated sequences of the *hsp65*, *rpoB* and 16S rRNA genes (Fig. 2), strains SN 1900^T
143 and SN 1904^T formed a well supported subclade that was sharply separated from an adjacent
144 taxon that encompassed the type strains of *Mycobacterium brumae* [39], *Mycobacterium*

145 *flavescens* [13], *Mycobacterium holsaticum* [40], *M. moriokaense* [33], *M. novocastrense* [14],
146 *Mycobacterium setense* [41] and *Mycobacterium vaccae* [38]. The genetic distances derived
147 from the multilocus sequence analyses between the test strains and their phylogenetic
148 neighbours were in very good agreement with the 16S rRNA gene data. Genetic distances of
149 0.107 and 0.106 were recorded between strains SN 1900^T and SN 1904^T and *M. novocastrense*
150 DSM 44203^T, values that rose to 0.117 and 0.118 against the *M. moriokaense* strain. It can be
151 concluded from all of these data that isolates SN 1900^T and SN 1904^T form a well supported
152 subclade within the evolutionary radiation occupied by rapidly growing mycobacteria, a
153 relationship that is underlined by the absence of 18 nucleotides at site 480 (*Escherichia coli*
154 16S rRNA gene position) in hypervariable region B of the 16S rRNA gene, this deletion is
155 known to be a characteristic feature of fast growing mycobacteria [42].

156 The genome sizes of strains SN1900^T and SN 1904^T were found to be ~5.5 Mb and ~5.4 Mb
157 with an average *in silico* G+C content of 66.7 mol% and 66.8 mol%, respectively. These
158 genomes are smaller than the ~6.2 Mb genome of *M. novocastrense* DSM 44203^T [43]. The
159 assembly size, coverage and contig numbers for strains SN 1900^T and SN1904^T were 5.5Mb
160 and 5.4MB; 47X and 81X, 76 and 81, respectively. The genomes of strains SN 1900^T and
161 SN1904^T were annotated using the RAST pipeline [44, 45] and shown to have 5,360 (51 RNAs)
162 and 5,154 (52 RNAs) coding sequences, respectively. The average nucleotide identity (ANI)
163 between the genomes of the test strains and the type strain of *M. novocastrense*, their close
164 phylogenetic neighbour, was calculated using the blastANI algorithm [46]. Digital DNA:DNA
165 hybridization (dDDH) values between isolates SN 1900^T, SN 1904^T and the *M. novocastrense*
166 strain were estimated using the GGDC based on formula 2 of Meier-Kolthoff *et al.* [23] which
167 is available on the DSMZ website (<http://ggdc.dsmz.de/>).

168 The dDDH relatedness between strains SN 1900^T and SN 1904^T was 61.0%, a value well below
169 the threshold of 70% for assigning bacterial strains to the same species [47]. The corresponding
170 ANI relatedness value between these strains was 94.7%, a cut off well below the threshold of
171 95-96 % used for prokaryotic species delineation [46, 48, 49]. In tum, the dDDH values
172 between strains SN 1900^T and SN 1904^T and the type strain of *M. novocastrense* were 41.4%
173 and 42.8 %, respectively, and the corresponding ANI values 89.3 % and 89.5 %.

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

177 procedure was used to obtain biomass for the fatty acid analyses but in this case cells were
178 grown in Middelbrook 7H10 broth. Standard thin-layer chromatographic procedures were used
179 to establish the chemotaxonomic profiles of strains SN 1900^T and SN 1904^T. To this end, they
180 were examined for isomers of diaminopimelic acid (A₂pm) [50]; predominant isoprenologues
181 [51, 52]; whole organism sugars [53]; and for polar lipids using the procedure of Minnikin *et*
182 *al.* [54]. The isolates were found to contain *meso*-A₂pm, MK-9(H₂) as the predominant
183 menaquinone (>95%), whole cell sugar profiles containing arabinose, galactose, glucose and
184 ribose, and to have polar lipid patterns consisting of diphosphatidylglycerol, glycerophospholipid,
185 phosphatidylethanolamine (PE), phosphatidylinositol, two aminolipids, a glycolipid and an
186 unidentified lipid; the *M. novocastrense* DSM 44203^T lacked PE and galactose.

187 Cellular fatty acids were extracted from the isolates and from *M. novocastrense* DSM 44203^T
188 and methylated according to Miller [55], as modified by Kuykendall *et al.* [56], then analysed
189 by gas chromatography (Agilent 6890N instrument). The resultant peaks were integrated and
190 the fatty acids identified using the standard Microbial Identification (MIDI) system, version 4.5
191 and the myco5 database [57]. Mycolic acid methyl esters prepared from these strains after
192 Minnikin and Goodfellow [58] were separated and identified, as described by Teramoto *et al.*
193 [59]. All three strains contained similar mixtures of saturated, unsaturated and 10-
194 methyloctadecanoic (tuberculostearic) fatty acids though quantitative differences were found
195 with respect to both major and minor components (Table 1). The major fatty acid (>25%)
196 detected in isolate SN 1904^T was C_{16:0} while isolate SN 1900^T and *M. novocastrense* DSM
197 44203^T contained a predominant proportion of fatty acids summed in feature 3 (Table 1).
198 Strains SN 1900^T and SN 1904^T contained dicarboxy mycolic acids with 61-64 carbon atoms
199 but *keto*-mycolates with 78-83 carbon atoms were found only in isolate SN 1904^T (Fig. S1).

200 The two test strains and *M. novocastrense* DSM 44203^T were examined for a broad range of
201 standard phenotypic procedures using media and methods described by Nouioui *et al.* [60];
202 these tests were carried out in triplicate using freshly prepared inocula (OD₆₀₀ 0.3-0.6)
203 harvested from the mid-logarithmic growth phase using PMG agar as the cultivation medium
204 and incubation at 37°C. Biochemical tests known to be of value in mycobacterial systematics
205 were performed on strains SN1900^T and SN1904^T, namely detection of arylsulfatase after 3
206 and 20 days [61], catalase [62], heat stable catalase [63], niacin accumulation [64] and nitrate
207 reductase [65]. The strains were also examined for the reduction of potassium tellurite [64, 66],
208 degradation of Tween 80 [67] and urea hydrolysis [62]. Additional enzymatic tests were
209 determined using API ZYM kits, as instructed by the manufacturer (Biomérieux, France). The
210 ability of the strains to oxidise carbon and nitrogen sources and to show resistance to inhibitory

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

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

229 It can be concluded from the wealth of phylogenetic, dDDH, chemotaxonomic and phenotypic
230 data that strains SN 1900^T and SN 1904^T can be distinguished from one another and from *M.*
231 *novocastrense* DSM 44203^T their close phylogenetic neighbour. Consequently, strains SN
232 1900^T and SN 1904^T are considered to represent two novel species within the genus
233 *Mycobacterium*, namely as *Mycobacterium lehmannii* sp. nov. and *Mycobacterium neumannii*
234 sp. nov., respectively.

235 A draft genome sequence is available for “*Mycobacterium acapulcensis*” CSURP1424, a strain
236 isolated from sputum of a patient with pulmonary lesions during a campaign against
237 tuberculosis [70]. However, this binomial name has not been validly published and hence has
238 no standing in nomenclature [71]. Strain CSURP1424 and SN 1900^T share a dDDH value of
239 81.3% and an ANI value of 98.3%, results well above the thresholds for assigning strains to the
240 same species [46-49]. Consequently, strain CSURP1424 is a *bona fide* member of the putative
241 novel species, *M. lehmannii*.

242 **Description of *Mycobacterium lehmannii* sp. nov.**

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

246 Gram-stain positive, acid-alcohol fast, facultatively anaerobic, non-motile, fast growing
247 organism which produces yellow-orange coloured colonies on Middelbrook 7H10, proteose
248 peptone-meat extract-glycerol, glucose-yeast extract-malt extract and tryptic soy agar plates
249 within 5 days at 37°C under dark and light conditions. Grows between 28°C and 37°C,
250 optimally ~ 37°C, and at pH7 and in the presence of 4% w/v sodium chloride. Produces
251 arylsulfatase within 3 and 20 days and reduces nitrate. Additional phenotypic tests are cited in
252 the text and in Table 2. *Meso*-diaminopimelic acid, arabinose, galactose, glucose and ribose are
253 present in whole organism hydrolysates and the predominant menaquinone is MK-9(H₂). The
254 polar lipid profile is composed of diphosphatidylglycerol, glycopospholipid,
255 phosphatidylethanolamine, phosphatidylinositol, two aminolipids and an unidentified lipid and
256 the major fatty acid (>20%) is summed feature 3. Contains dicarboxy mycolic acids with 61-
257 64 carbon atoms. The *in silico* DNA G+C content is 66.7%. The type strain is SN 1900^T (40^T =
258 CECT 8763^T = DSM 43219^T).

259

260 **Description of *Mycobacterium neumannii* sp. nov.**

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

264 Gram-stain positive, acid-alcohol fast, facultatively anaerobic, non-motile, fast growing
265 organism which produces yellow-orange coloured colonies on Middelbrook 7H10, proteose
266 peptone-meat extract-glycerol, glucose-yeast extract-malt extract and tryptic soy agar plates
267 within 5 days at 37°C under dark and light conditions. Grows between 28°C and 37°C,
268 optimally ~37°C, at pH7 and in the presence of up to 8% w/v sodium chloride. Produces
269 arylsulfatase after 3 and 20 days but not nitrate reductase. Additional phenotypic tests are cited
270 in the text and in Table 2. *Meso*-diaminopimelic acid, arabinose, galactose, glucose and ribose
271 are present in whole organism hydrolysates and the predominant menaquinone is MK-9(H₂).
272 The polar lipid profile consists of diphosphatidylglycerol, glycopospholipid,
273 phosphatidylethanolamine, phosphatidylinositol, two aminolipids, glycolipid and an
274 unidentified lipid and the major fatty acid (>20%) is C_{16:0}. Contains dicarboxy mycolic acids

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

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281

282 **Conflicts of interest**

283 The authors declare that they have no conflicts of interest.

284

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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	<i>M. novocastrense</i> 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	<i>M. novocastrense</i> 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	-	-	+
<i>N</i> -acetyl-D-Glucosamine	-	+	+
Utilisation of amino acids :			
Glucuronamide	-	+	-
Glycine-proline	+	-	-
D-Serine	+	+	-
Utilisation of organic acids:			
γ -amino- <i>n</i> -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.

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

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489 **Figure legends**

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491 **Fig. 1.** Maximum-likelihood phylogenetic tree based on almost complete 16S rRNA gene
 492 sequences inferred using the GTR+GAMMA model and rooted by midpoint-rooting. The
 493 numbers above the branches are bootstrap support values greater than 60% for ML (left) and
 494 MP (right).

495 **Fig. 2.** Maximum-likelihood MLST phylogenetic tree constructed from 2332 nucleotide
 496 concatenated sequences of 16S rRNA, *hsp65* and *rpoB* genes. Bootstrap values above 50% are
 497 displayed.

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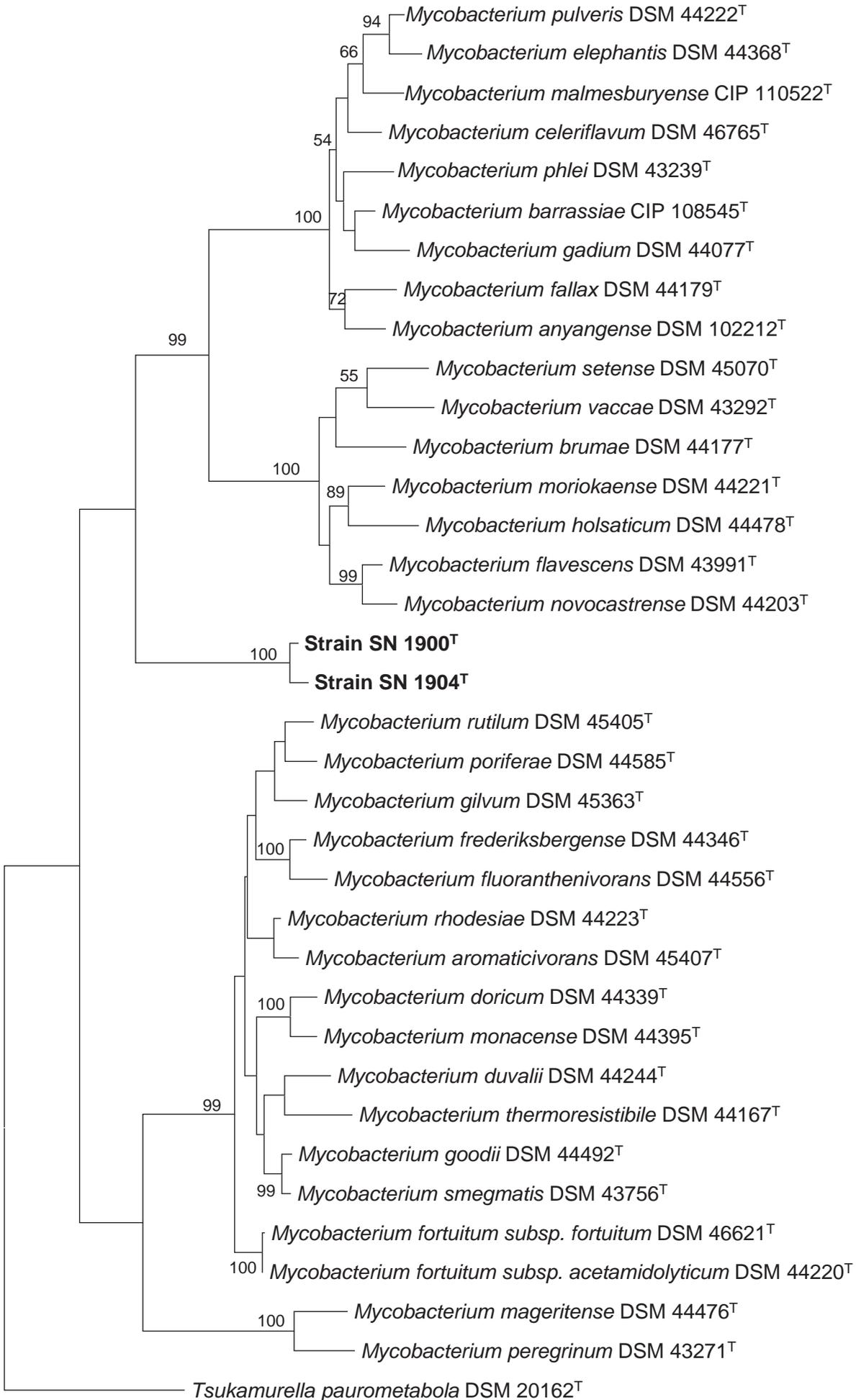
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Figure

60 LFN KH UH WR GRZQ ID



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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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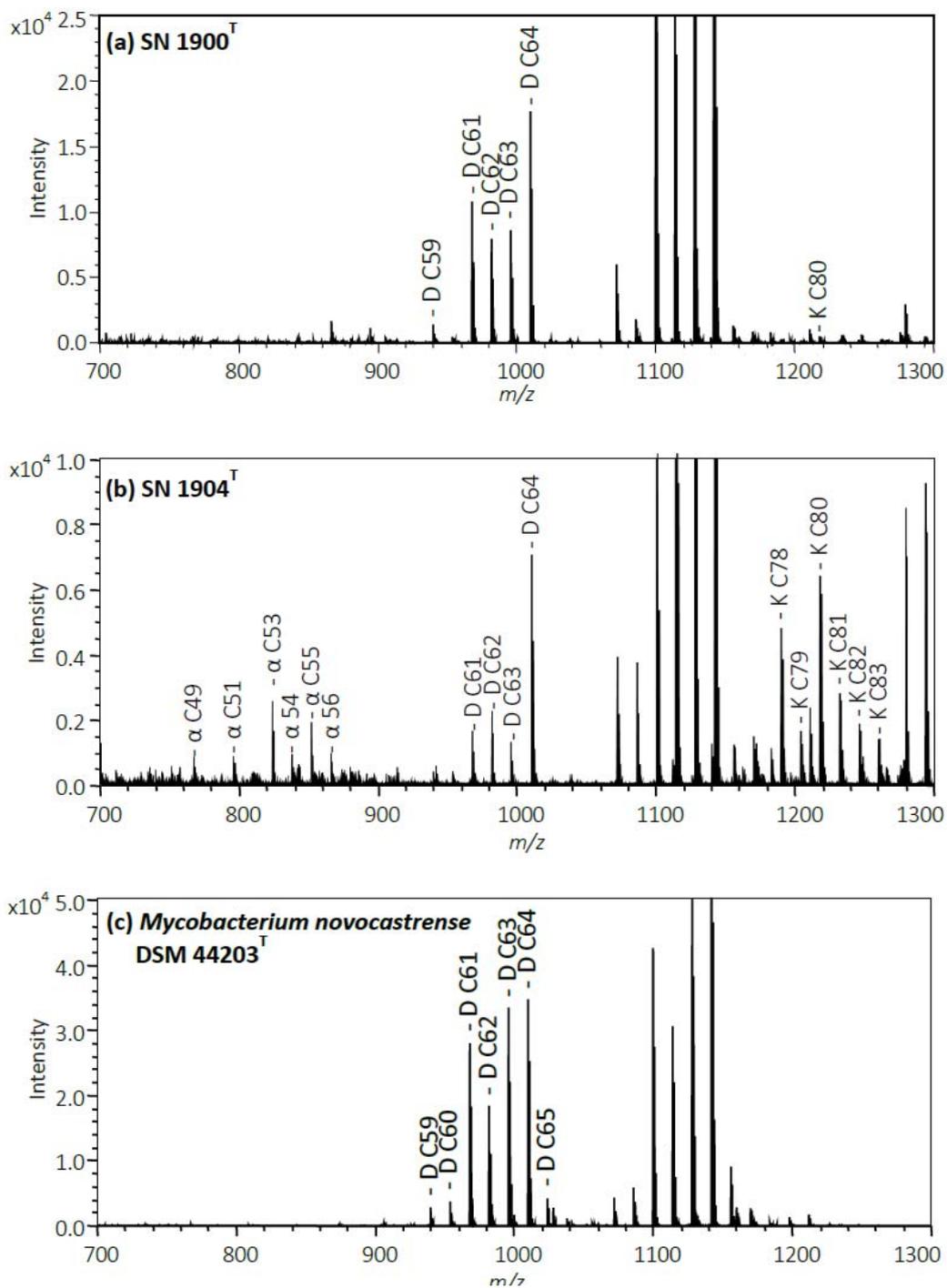


Fig. S1. Mycolic acid profiles of strains SN1900^T (a), SN1904^T (b) and *M. novocastrense* DSM 44203^T (c). D = dicarboxy-mycolic acid; α = α -mycolic acid, K= keto-mycolic acid.