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28

Abstract

29 An actinomycete, strain N1286^T, isolated from a lung transplant patient with a
30 pulmonary infection, was provisionally assigned to the genus *Nocardia*. The strain had
31 chemotaxonomic and morphological properties typical of members of the genus
32 *Nocardia* and formed a distinct phyletic line in the *Nocardia* 16S rRNA gene tree. It
33 was most closely related to *Nocardia farcinica* DSM 43665^T (99.8% gene similarity)
34 but was distinguished from the latter by a low level of DNA:DNA relatedness. These
35 strains were also distinguished by a broad range of phenotypic properties. On the
36 basis of these data, it is proposed that isolate N1286^T (=DSM 45810^T = NCTC
37 13617^T) should be classified as the type strain of a new *Nocardia* species for which
38 the name *Nocardia kroppenstedtii* is proposed.

39

40 Improvements in the classification of the genus *Nocardia* due to the application of
41 polyphasic taxonomy provide a sound framework for the recognition of additional
42 species (Goodfellow & Maldonado, 2012). At the time of writing, the genus
43 encompasses 85 validly published species (<http://www.bacterio.net/n/nocardia.html>),
44 including the recently described *Nocardia grenadensis* (Kämpfer *et al.*, 2012),
45 *Nocardia rhamnosiphila* (Everest *et al.*, 2011), *Nocardia goodfellowii* and *Nocardia*
46 *thraciensis* (Sazak *et al.*, 2012). Nocardiae form a clade within the evolutionary
47 radiation occupied by mycolic acid-containing actinomycetes, that is, microorganisms
48 belonging to genera assigned to the order *Corynebacteriales* (Goodfellow & Jones,
49 2012). Most recently described *Nocardia* species are associated with human
50 infections (Brown-Elliott *et al.*, 2006; Goodfellow & Maldonado, 2012), as
51 exemplified by *Nocardia mikamii* (Jannat-Khah *et al.*, 2010) and *Nocardia niwae*
52 (Moser *et al.*, 2011). Here we describe the results of phenotypic and phylogenetic

53 analyses of another strain isolated from clinical material and show that it represents a
54 new *Nocardia* species.

55

56 Strain N1286^T was isolated from bronchial lavage cultured on chocolate agar
57 incubated at 37°C in 5% CO₂ for 2 days. The organism was maintained on glucose-
58 yeast extract agar (GYEA; Gordon & Mihm, 1962) at room temperature and as
59 glycerol suspensions (20%, v/v) at -20°C, as were *Nocardia asteroides* DSM 43757^T
60 and *Nocardia farcinica* DSM 43665^T. Biomass of all strains analysed, for the
61 chemotaxonomic and molecular systematic studies was grown in shake flasks of GYE
62 broth for 5 days at 28°C, checked for purity and harvested by centrifugation. Cells for
63 the chemosystematic analyses were washed twice in distilled water and freeze-dried;
64 those for the molecular systematic work were washed in NaCl/EDTA buffer (0.1M
65 EDTA, 0.1M NaCl, pH 8.0) and stored at -20°C until required.

66

67 The phylogenetic position of isolate N1286^T was determined by 16S rRNA gene
68 sequence analysis. Chromosomal DNA was isolated, PCR fragments amplified and
69 direct sequencing of the purified products carried out after Kim *et al.*, (1998). The
70 almost complete 16S rRNA gene sequence (1544 nucleotides [nt]) was aligned
71 manually against corresponding sequences of genera classified in the order
72 *Corynebacteriales*, retrieved from the DDBJ/EMBL/GenBank databases, using the
73 pairwise alignment option and 16S rRNA secondary structural information held in the
74 MEGA5 program (Tamura *et al.*, 2011). Phylogenetic trees were inferred using the
75 maximum-parsimony (Kluge & Farris, 1969), maximum-likelihood (Felsenstein,
76 1981) and neighbour-joining (Saitou & Nei, 1987) tree-making algorithms from the
77 MEGA5 software. The Jukes and Cantor (1969) model was used to generate an

78 evolutionary distance matrix for the neighbour-joining algorithm. Topologies of the
79 resultant unrooted trees were evaluated by bootstrap analysis of the neighbour-joining
80 method (Felsenstein, 1985) based upon 1000 replicates using MEGA 5 software.

81

82 It can be seen from Figures 1 and S2, that strain N1286^T formed a distinct subclade in
83 the 16S rRNA *Nocardia* gene tree together with the type strain of *N. farcinica*, an
84 association supported by all of the tree-making algorithms and by a 99% bootstrap
85 value in the neighbour-joining analysis. The strains shared a 16S rRNA gene
86 similarity of 99.8%, a value that corresponded to 3 nt differences at 1544 locations.
87 The two strains were associated with the type strains of *Nocardia higoensis* and
88 *Nocardia shimofuensis*, as shown in Figure 1; strain N1286^T shared 16S rRNA
89 similarities of 98.9% with the *N. higoensis* and *N. shimofuensis* strains, a value
90 equivalent to 17 nt differences.

91

92 Strain N1268^T was examined for key chemotaxonomic markers considered to be
93 characteristic of *Nocardia* strains using *N. asteroides* DSM 43757^T as control.
94 Standard procedures were used to determine the diagnostic isomers of diaminopimelic
95 acid (A₂pm; Stanek & Roberts, 1974), cellular fatty acids (Sutcliffe, 2000),
96 isoprenoid quinones (Collins, 1994), muramic acid type (Uchida *et al.*, 1999), mycolic
97 acids (Minnikin *et al.*, 1975), polar lipids (Minnikin *et al.*, 1984) and whole-organism
98 sugars (Hasegawa *et al.*, 1983). The organism contained *meso*-A₂pm, arabinose and
99 galactose, in whole-organism hydrolysates (wall chemotype IV sensu, Lechevalier &
100 Lechevalier, 1970); N-glycolyl muramic acid; hexahydrogenated menaquinone with
101 eight isoprene units where the two end units were cyclized (MK- 8 [H₄], ω cyclo) as
102 the sole isoprenologue; major proportions of straight chain saturated, unsaturated and

103 tuberculostearic acids (fatty acid type 1b *sensu*, Kroppenstedt, 1985),
104 diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol
105 phosphatidylinositolmannosides as major polar lipids (Fig S1.); and mycolic acids
106 that co-migrated with those from the type strain of *N. asteroides*. This
107 chemotaxonomic profile is consistent with the classification of isolate N1268^T in the
108 genus *Nocardia* (Goodfellow & Maldonado, 2012).

109

110 DNA:DNA relatedness values (ΔT_m) were determined, in triplicate, between isolate
111 N1286^T and *N. farcinica* DSM 43665^T using the fluorimetric method described by
112 Gonzalez and Sait-Jimenez (2005); the optimum temperatures for reassociation (T_{or})
113 were calculated using the equation $T_{or} = 0.51 (\%GC) + 47$. The melting temperatures
114 (T_m) at which 50% of the initial double-stranded DNA molecules denatured into
115 single-stranded DNA for isolate N1286^T g DNA and isolate N1286^T / *N. farcinica*
116 hybrid DNA preparations were compared and the differences (ΔT_m) calculated. The
117 %GC was 80.2%, the mean ΔT_m between isolate N1286^T g DNA and isolate N1286^T/
118 *N. farcinica* hybrid DNA was $9.6 \pm 1.2^\circ\text{C}$, a value which represents a DNA:DNA
119 relatedness value of $44 \pm 4\%$ (Gonzalez & Saiz-Jimenez, 2005).

120

121 Isolate N1286^T and the type strain of *N. farcinica*, were examined for a range of
122 phenotypic properties using well established media known to be of value in nocardial
123 systematics (Andrews, 2001; Goodfellow, 1971; Goodfellow & Maldonado, 2012;
124 Isik *et al.*, 1999). A number of differential characteristics separated the two strains;
125 isolate N1286^T, unlike the *N. farcinica* strain, grew at 37°C, did not produce aerial
126 mycelium, degraded starch, hydrolysed aesculin and arbutin; grew on *meso*-inositol
127 and methyl- α -D-glucopyranoside as a sole carbon source (1% w/v) and was not

128 inhibited by bacitracin (10 units). Similarly, *N. farcinica* DSM 43665^T, unlike the
129 isolate, degraded DNA, and RNA; reduced nitrate, and grew on dulcitol and *i*-
130 erythritol (1% w/v) and on sodium benzoate, oxalic acid and pimelic acid (0.1% w/v)
131 as sole carbon sources and in the presence of fusidic acid (10 µg).

132

133 It can be concluded that isolate N1286^T forms a distinct phyletic line in the *Nocardia*
134 16S rRNA gene tree and can be distinguished readily from *N. farcinica* DSM 43665^T,
135 its nearest phylogenetic neighbour, using a combination of phenotypic features.
136 Consequently, it is proposed that isolate N1286^T should be recognised as a new
137 species, *Nocardia kroppenstedtii*.

138

139 **Description of *Nocardia kroppenstedtii* sp. nov.**

140 *Nocardia kroppenstedtii* (krop. pen. sted'ti.i. N.L. n. *kroppenstedtii*, of Kroppenstedt
141 to honour Reiner Kroppenstedt, a German microbiologist, for his many contributions
142 to actinobacterial systematics).

143

144 Aerobic, Gram-positive, nonmotile, nonsporeforming, partially-acid alcohol fast,
145 catalase-positive, actinomycete which forms a mycelium that fragments into rods and
146 cocci. Irregular, wrinkled, matt, pale orange yellow pigmented colonies are formed on
147 modified Bennett's agar after 5 days growth at 30°C. Growth occurs at pH 6.0-10.0,
148 from 25°C to 37°C and optimally ~ 28°C. Uric acid is not degraded. D-arabitol,
149 arbutin, D-fucose, glycerol and D-ribose (1%, w/v), *n*-propanol (1%, v/v) and γ -
150 hydroxybutyric acid, sodium fumarate, sodium-DL-malate and sodium suberate
151 (0.1%, w/v) are used as sole carbon sources. Growth occurs in the presence of filter
152 paper discs soaked in bacitracin (10 units), cephalexin (30 µg), clindamycin

153 hydrochloride (2 µg), colistin (25 µg), cotrimoxazole (25 µg), erythromycin (5 µg),
154 nalidixic acid (30 µg), novobiocin (5 µg), penicillin (1 µg) and tetracycline
155 hydrochloride (10 µg), but not in the presence of discs soaked in ciprofloxacin (1 µg)
156 and fusidic acid (10 µg). Additional phenotypic properties are cited in the text. The
157 major cellular fatty acid components are hexadecanoic (C16:0; 30.8 %),
158 monosaturated hexadecanoic (C16:1; 18.6 %), octadecanoic (C18:0; 7.2 %),
159 monosaturated octadecanoic (C18:1; 6.4 %), tuberculostearic acid (TSA₁₈; 30.2 %)
160 and eicosanoic (C 20:0; 5.2 %). Additional chemotaxonomic properties are also
161 typical of nocardiae.

162

163 The type strain, N1286^T (=DSM 45810 = NCTC 13617^T), was isolated from a lung
164 transplant patient with a pulmonary infection. The species description is based on a
165 single strain and hence serves as a description of the type strain.

166

167 **Acknowledgements**

168

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171 gene of strain N1286^T, respectively.

172

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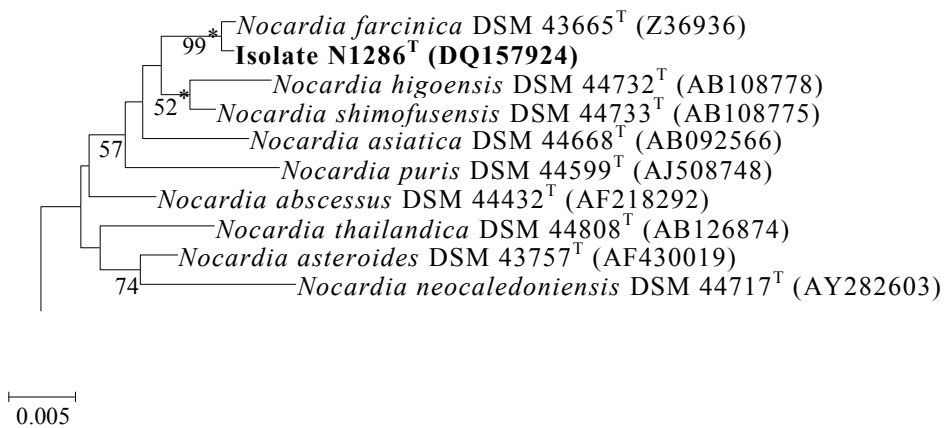


Fig.1. A section of the neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the position of strain N1286^T relative to its nearest neighbours. Asterisks indicate branches of the tree that were also found with the maximum-likelihood and maximum-parsimony tree-making algorithms; L and M indicate branches found using the maximum-likelihood and maximum-parsimony methods, respectively. The numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1000 re-sampled datasets; only values above 50% are given. The scale bar indicates 0.005 substitutions per nucleotide position. ^T, type strain.

284 Supplementary figures

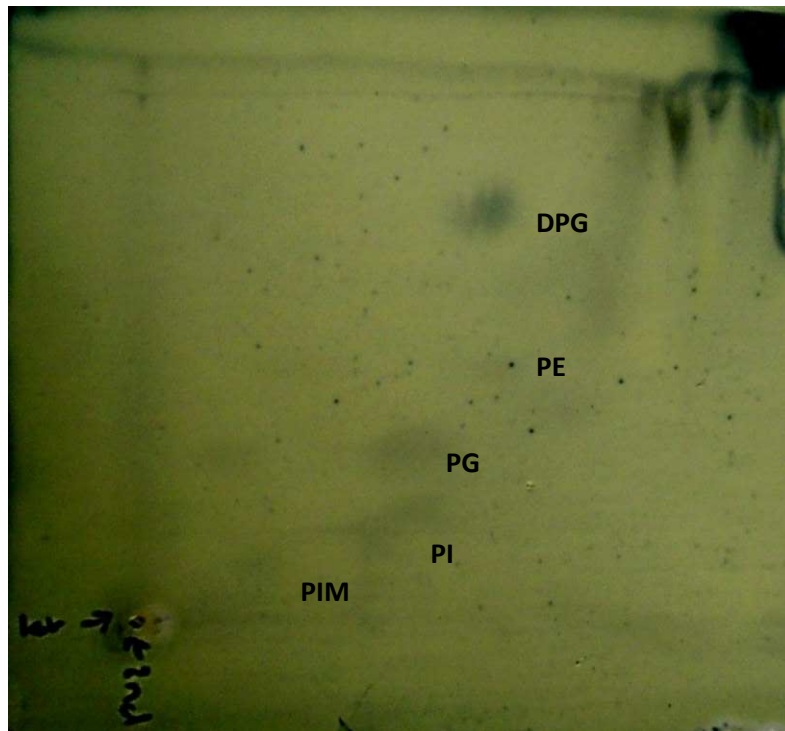
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286 Fig. S1: Polar lipid composition of strain N1286^T. The polar lipids were identified as follows: DPG,
287 diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI,
288 phosphatidylinositol; PIM, phosphatidylinositolmannosides.

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295 Fig. S2. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the
296 position of strain N1286^T. Asterisks indicate branches of the tree that were also found with the
297 maximum-likelihood and maximum-parsimony tree-making algorithms; L and M indicate branches
298 found using the maximum-likelihood and maximum-parsimony methods, respectively. The numbers at
299 the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1000 re-
300 sampled datasets; only values above 50% are given. The scale bar indicates 10 substitutions per
301 nucleotide position. ^T, type strain.

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