***Nocardioides albertani*, sp. nov., isolated from Roman catacombs[◇]**

Cynthia Alias-Villegas^{1†}, Valme Jurado^{1*†}, Leonila Laiz¹, Ana Z. Miller²,
Cesareo Saiz-Jimenez¹

¹Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, Av. Reina
Mercedes 10, 41012 Sevilla, Spain

²Centro de Petrologia e Geoquímica. Instituto Superior Técnico, Av. Rovisco
Pais, 1049-001, Lisboa, Portugal

[◇]This paper is dedicated to the memory of Professor Patrizia Albertano who
died on March 14th, 2012

† These authors contributed equally to this work.

* Corresponding author:

Valme Jurado,

Instituto de Recursos Naturales y Agrobiología, CSIC,

Apartado 1052, 41080 Sevilla, Spain

Tel. +34 95 462 4711, Fax +34 95 462 4002

E-mail: vjurado@irnase.csic.es

Keywords: *Nocardioides albertani*, Roman catacombs, rRNA, sequence

The sequence of the 16S rRNA gene from strain CD40127^T can be accessed at
Genbank accession number HE801966.

26 A Gram-positive, aerobic, non-spore-forming, rod- or coccoid-shaped, strain
27 CD40127^T, was isolated from a green biofilm covering the wall of the Domitilla
28 Catacombs in Rome, Italy. Phylogenetic analysis based on 16S rRNA gene
29 sequences revealed that strain CD40127^T belongs to the genus *Nocardioides*,
30 closely related to *Nocardioides luteus* DSM 43366^T and *Nocardioides albus*
31 DSM 43109^T with 98.86% and 98.01% similarity values, respectively. Strain
32 CD40127^T exhibited 16S rRNA gene sequence similarity values below 96.29%
33 with the rest of *Nocardioides* species. The G+C content of the genomic DNA
34 was 69.7 mol%. The predominant fatty acid was iso-C_{16:0} and the major
35 menaquinone was MK-8 (H₄) according to the genus *Nocardioides*. A
36 polyphasic approach using physiological tests, fatty acid profiles, DNA base
37 ratios and DNA-DNA hybridization showed that isolate CD40127^T belongs to a
38 novel species within the genus *Nocardioides*, for which the name *Nocardioides*
39 *albertani* is proposed. The type strain is CD40127^T (=DSM 25218^T =CECT
40 8014^T).

41

42 The genus *Nocardioides* was established by Prauser (1976) with *Nocardioides*
43 *albus* for Gram-positive, nonacid-fast, catalase positive, aerobic, and mesophilic
44 nocardioform actinomycetes, developing a mycelium that fragments into
45 irregular rod- to coccus-like elements. Currently, the genus *Nocardioides*
46 comprises 57 species with validly published names
47 (<http://www.bacterio.cict.fr/n/nocardioides.html>) and isolated from a variety of
48 habitats, including soils, sediments, sand, water, herbage, an oil shale column,
49 and glacier cryoconite and many of these were isolated recently from samples
50 collected in Korea (Dastager *et al.*, 2008, 2010; Yoon *et al.*, 1997, 2009, 2010).
51 In addition, strains of the genus *Nocardioides* have been isolated from caves in
52 Spain and Italy (Groth *et al.*, 1999, 2001), but the authors were unable to assign
53 the isolates to a defined species.

54

55 During the investigations on the microbial biodiversity from Roman catacombs
56 (<http://www2.bio.uniroma2.it/biologia/laboratori/lab-botanica/Algae/CATS.htm>) a
57 large number of new species of actinobacteria were found. In this study, we
58 describe strain CD40127^T isolated from a green biofilm, mainly composed of
59 phototrophic microorganisms, covering a wall of Domitilla Catacombs in Rome,
60 Italy. A polyphasic approach showed that the isolate represents a novel species
61 within the genus *Nocardioides*.

62

63 Strain CD40127^T was isolated on casein agar media (Küster & Williams, 1964)
64 after one week incubation at 28°C. Morphological, physiological and
65 chemotaxonomic studies were carried out using cultures on trypticase soy agar
66 (TSA; Oxoid) at 28°C except indicated otherwise. Cell morphology and

67 dimensions were observed by a stereo microscope and phase contrast
68 microscope. Media such as oatmeal agar (Prauser, 1976), nutrient agar (Difco)
69 and R2A agar (Difco) were used for testing mycelial production. Oxidase activity
70 was determined by monitoring the oxidation of dryslide oxidase (Becton
71 Dickinson). Catalase production was indicated by the production of bubbles
72 after mixing a cell suspension with a drop of 3% hydrogen peroxide solution on
73 a slide. Acid production from a variety of substrates was tested using the API 50
74 CH system and API 50 CH B/E kit (bioMérieux). Assimilation tests were carried
75 out using the API 20NE kit (bioMérieux) and enzymatic activities were detected
76 with API ZYM galleries (bioMérieux). All API tests were performed according to
77 the manufacturer's instructions. For the Gram reaction, a 3% solution of
78 potassium hydroxide was used (Haleblian *et al.*, 1981). Growth temperature was
79 tested over the range 4-45°C. Tolerance to NaCl was studied on TSA
80 supplemented with 0-15% (w/v) NaCl.

81

82 Standard procedures for the analyses of fatty acids by gas chromatography
83 were adopted with the Microbial Identification System (MIDI) for automated GC
84 analyses (Kroppenstedt, 1985) using TSA after 3 days at 28°C. Analysis of
85 respiratory quinones and G+C content of genomic DNA were determined by the
86 DSMZ, Germany.

87

88 Genomic DNA extraction was performed as described by Marmur (1961). The
89 16S rRNA gene was amplified by PCR using the primers 27F (5'-
90 AGAGTTTGATCCTGGCTCAG) and 1522R (5'-
91 AAGGAGGTGATCCAGCCGCA). PCR thermal conditions were as follows:

92 95°C for 60 s; 35 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 120 s; and a
93 final extension cycle at 72 °C for 10 min. Forward and reverse strands of the
94 amplified DNA fragment were sequenced in an ABI 3700 sequencer (Applied
95 Biosystems). The identification of phylogenetic neighbours was carried out by
96 submitting the sequence of the strain CD40127^T in BLAST (Altschul *et al.*, 1990)
97 and by using GenBank database and the EzTaxon-e database (Kim *et al.*,
98 2012). Pairwise 16S rRNA gene sequence similarities among the most closely
99 related strains were determined using the global alignment algorithm on the
100 EzTaxon server (<http://eztaxon-e.ezbiocloud.net/>) (Kim *et al.*, 2012). For
101 phylogenetic analyses, the nearly complete 16S rRNA gene sequence (1379 nt)
102 of strain CD40127^T was aligned and compared with corresponding sequences
103 of members of the genus *Nocardioides* using the multiple sequence alignment
104 program MUSCLE (Edgar, 2004) integrated in MEGA 5 software. Phylogenetic
105 and molecular evolutionary analyses were conducted using MEGA version 5
106 (Tamura *et al.*, 2011) and by applying the neighbour-joining (Saitou & Nei,
107 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein,
108 1981) algorithms in MEGA 5 software. Tree robustness was assessed by
109 bootstrap resampling (1,000 replicates each). The degree of genomic
110 relatedness among strain CD40127^T, *Nocardioides albus* DSM 43109^T and
111 *Nocardioides luteus* DSM 43366^T, which shared high similarity values for their
112 16S rRNA gene sequence, was determined by DNA–DNA hybridization as
113 described by De Ley *et al.* (1970) and Rosselló-Mora & Amann (2001).

114

115 Cells of strain CD40127^T were aerobic, Gram-positive, catalase-positive and
116 oxidase-negative, non-spore-forming and rod-shaped or coccoid. Growth of

117 strain CD40127^T occurred in the temperature range of 10-30°C, with an
118 optimum at 25°C. Strain CD40127^T grew at NaCl concentrations of 0-10% (w/v)
119 (optimum 0-4%). Table 1 shows other physiological characteristics of strain
120 CD40127^T, as well as numerous phenotypic differences from the
121 phylogenetically closest species of the *Nocardioides* genus. Several
122 physiological and chemotaxonomic differences were noted among strains
123 CD40127^T, *N. albus* DSM 43109^T and *N. luteus* DSM 43366^T. These
124 differences included the growth at 37°C and spore production. Strain CD40127^T
125 did not grow at 37°C and either did not produce spore on the tested culture
126 media, while *N. albus* and *N. luteus* grew well at this temperature and produced
127 spores. Other differences were the production of acid from L-rhamnose and the
128 presence or absence of gelatinase, α -mannosidase and trypsin activities.
129 Assimilation of N-acetylglucosamine and maltose differed among the three
130 strains. Further dissimilarities were noticed in fatty acid composition. Although
131 14-methyl pentadecanoic acid (iso-C_{16:0}) is the predominant fatty acid in all
132 three *Nocardioides* species, there were differences in the abundance of fatty
133 acids; C_{18:0} 10-methyl, C_{18:1} ω 9c and C_{17:0} 10-methyl (Supplementary Table S1).
134 The menaquinone pattern revealed that MK-8 (H₄) was the predominant
135 isoprenoid quinone (76%) according with the genus *Nocardioides*; MK-8 (H₂)
136 was present as a minor component (24%).

137

138 Phylogenetic analysis showed that strain CD40127^T was related with the genus
139 *Nocardioides*. According to the 16S rRNA gene sequence similarity, strain
140 CD40127^T was most closely related to *N. luteus* DSM 43366^T (GenBank
141 accession number AF005007) and *N. albus* DSM 43109^T (AF004988) with

142 similarity values of 98.86% and 98.01%, respectively. In the phylogenetic tree
143 based on the 16S rRNA gene sequence (Fig. 1), strain CD40127^T formed a well
144 defined clade with the type strains of *N. luteus* and *N. albus* that is supported by
145 a bootstrap value of 100% in the neighbour-joining analysis. Strain CD40127^T
146 showed DNA-DNA relatedness of 48.90% with *N. luteus* DSM 43366^T and
147 58.79% with *N. albus* DSM 43109^T. These results indicate that strain CD40127^T
148 shows sufficient genomic coherence and hybridization differences from its
149 closest relatives to be considered as a single species (Roselló-Mora & Amann,
150 2001; Stackebrandt *et al.*, 2002).

151

152 Phenotypic and genotypic characteristics described above and in the species
153 description below, together with the differences observed between strain
154 CD40127^T and previously described species of the genus *Nocardioides* reveal
155 that strain CD40127^T is a novel species within the genus *Nocardioides*. The
156 name *Nocardioides albertani* sp. nov. is proposed for this novel species.

157

158

159

160 **Description of *Nocardioides albertani* sp. nov.**

161

162 *Nocardioides albertani* (al.ber.ta'ni. N.L. gen. fem. *albertani* named in
163 honour of Prof. Patrizia Albertano).

164

165 Cells are Gram-positive, aerobic, non-spore-forming, non-motile and rod-shape
166 or coccoid (0.6-0.8 µm wide and 1.0-1.6 µm long after 2 days on R2A agar).

Colonies on R2A agar are cream-coloured, smooth, circular and 0.1 mm in diameter after 2 days growth at 28°C on R2A agar. Neither substrate nor aerial mycelium is formed. Catalase-positive and oxidase-negative. It does not reduce nitrate to nitrite. Growth occurs between 10 and 30°C, optimum at 25°C. Cells grow at NaCl concentrations of 0-10% (w/v) (optimum 0-4%). It does not produce indole. It produces acid from aesculin. Assimilates arabinose, glucose, mannose, mannitol, N-acetyl-glucosamine, maltose, potassium gluconate and malate, but does not assimilate capric acid, adipic acid, trisodium citrate and phenylacetic acid. Produces acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), β -galactosidase, gelatinase, α,β -glucosidase, α -mannosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase but not arginine dihydrolase, α -chymotrypsin, α -fucosidase, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, lipase (C14), trypsin and urease. Variable glucose fermentation activity. The predominant fatty acid is iso-C_{16:0}. The G+C content of the type strain is 69.7 mol%. The major menaquinone is MK-8 (H₄).

The type strain, CD40127^T (=DSMZ 25218^T = CECT 8014^T) was isolated from a green biofilm covering the wall of the Domitilla Catacombs, Rome, Italy.

Acknowledgements

This work was supported by Consolider project TCP CSD2007-00058 and Portuguese Funds through FCT – *Fundação para a Ciência e a Tecnologia* with a postdoctoral fellowship (SFRH/BPD/63836/2009).

192 **References**

193

194 **Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990).** A
195 basic local alignment search tool. *J Mol Biol* **215**, 403–410.

196 **Dastager, S. G., Lee, J. C., Ju, Y. J., Park, D. J. & Kim, C. J. (2008).**
197 *Nocardioides koreensis* sp. nov., *Nocardioides bigeumensis* sp. nov. and
198 *Nocardioides agariphilus* sp. nov., isolated from soil from Bigeum Island, Korea.
199 *Int J Syst Evol Microbiol* **58**, 2292-2296.

200 **Dastager, S. G., Lee, J. C., Pandey, A. & Kim, C. J. (2010).** *Nocardioides*
201 *mesophilus* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **60**, 2288-2292.

202 **De Ley, J., Cattoir, H. & Reynaerts, A. (1970).** The quantitative measurement
203 of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

204 **Edgar, R. C. (2004).** MUSCLE: multiple sequence alignment with high accuracy
205 and high throughput. *Nucleic Acids Res* **32**, 1792-1797.

206 **Felsenstein, J. (1981).** Evolutionary trees from DNA sequences: a maximum
207 likelihood approach. *J Mol Evol* **17**, 368-376.

208 **Fitch, W. M. (1971).** Toward defining the course of evolution: minimum change
209 for a specific tree topology. *Syst Zool* **20**, 406–416.

210 **Groth, I., Vettermann, R., Schuetze, B., Schumann, P. & Saiz-Jimenez, C.**
211 **(1999).** Actinomycetes in Karstic caves of northern Spain (Altamira and Tito
212 Bustillo). *J Microbiol Meth* **36**, 115-122.

213 **Groth, I., Schumann, P., Laiz, L., Sanchez-Moral, S., Cañaveras, J.C. &**
214 **Saiz-Jimenez, C. (2001).** Geomicrobiological Study of the Grotta dei Cervi,
215 Porto Badisco, Italy. *Geomicrobiol J* **18**, 241-258.

216 **Haleblian, S., Harris, B., Finegold, S.M. & Rolfe, R.D. (1981).** Rapid method
217 that aids in distinguishing Gram-positive from Gram negative anaerobic bacteria.
218 *J Clin Microbiol* **13**, 444–448.

219 **Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C.,**
220 **Jeon, Y. S., Lee, J. H., Yi, H., Won, S. & Chun, J. (2012).** Introducing
221 EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes
222 that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

223 **Kroppenstedt, R. M. (1985).** Fatty acid and menaquinone analysis of
224 actinomycetes and related organisms. In *Chemical Methods in Bacterial*
225 *Systematics* (Society for Applied Bacteriology Technical Series vol. 20), pp.
226 173–199. Edited by M. Goodfellow & D. E. Minnikin. New York: Academic
227 Press.

228 **Küster, E. & Willians, S.T. (1964).** Selection of media for isolation of
229 streptomycetes. *Nature* **202**, 928–929.

230 **Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from
231 microorganisms. *J Mol Biol* **3**, 208–218.

232 **Prauser, H. (1976).** *Nocardioide*s, a new genus of the order *Actinomycetales*.
233 *Int J Syst Bacteriol* **26**, 58-65.

234 **Rosselló-Mora & Amann (2001).** The prokaryotes. *FEMS Microbiol Rev* **25**,
235 39–67.

236 **Saitou, N. & Nei, M. (1987).** The neighbour-joining method: a new method for
237 reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.

238 **Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D.,**
239 **Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. &**

240 **other authors (2002).** Report of the ad hoc committee for the re-evaluation of
241 the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

242 **Tamura, K., Peterson, D., Peterson, N., Stecher, G, Nei, M., Kumar, S.**
243 **(2011).** MEGA 5: Molecular Evolutionary Genetics Analysis using Maximum
244 Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol Biol*
245 *Evol* **28**: 2731-2739.

246 **Yoon, J. H., Rhee, S. K., Lee, J. S., Park, Y. H. & Lee, S. T. (1997).**
247 *Nocardioides pyridinolyticus* sp. nov., a pyridine-degrading bacterium isolated
248 from the oxic zone of an oil shale column. *Int J Syst Bacteriol* **47**, 933-938.

249 **Yoon, J. H., Kang, S. J., Park, S., Kim, W. & Oh, T. K. (2009).** *Nocardioides*
250 *caeni* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **59**, 2794-
251 2797.

252 **Yoon, J. H., Park, S., Kang, S. J., Lee, J. S., Lee, K.C. & Oh, T. K. (2010).**
253 *Nocardioides daedukensis* sp. nov., a halotolerant bacterium isolated from soil.
254 *Int J Syst Evol Microbiol* **60**, 1334-1338.

255

Table 1. Phenotypic characteristics of strain CD40127^T and related species

Tested strains were all negative for nitrate reduction and indole production. All strains assimilated arabinose, glucose, mannose, mannitol, potassium gluconate and malate, and they did not assimilate capric acid, adipic acid, trisodium citrate and phenylacetic acid. All strains had catalase, acid and alkaline phosphatases, cystine arylamidase, esterase (C4), esterase lipase (C8), β -galactosidase, α,β -glucosidases, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities; none of them had oxidase, arginine dihydrolase, α -chymotrypsin, α -fucosidase, α -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, lipase (C14) and urease. All strains were grown under the same conditions for all results presented in this table. +, positive; -, negative; v, variable; w, weakly positive

Characteristic	Strain CD40127 ^T	<i>Nocardioides albus</i> DSM 43109 ^T	<i>Nocardioides luteus</i> DSM 43366 ^T
Cell morphology	Rods, cocci	Hyphae	Hyphae
Cell length (μ m)	1.0-1.6	v	v
Cell width (μ m)	0.6-0.8	0.5-1.0	0.5-1.0
Colony colour	cream	cream	Yellow to cream
Growth at 37°C	-	+	+
Acid produced from L-rhamnose	-	+	-
Enzyme activities:			
Gelatinase	+	+	-
α -Mannosidase	+	-	+
Trypsin	-	w	+
Assimilation of:			
N-acetyl-glucosamine	+	-	+
Maltose	+	-	+
Major fatty acids (>5%)	iso-C _{16:0}	iso-C _{16:0} C _{18:0} 10-methyl C _{18:1} ω 9c	iso-C _{16:0} C _{17:0} 10-methyl
Predominant menaquinone	MK8 (H ₄)	MK8 (H ₄)	MK8 (H ₄)
G+C content	69.7 mol%	66.5-68.6 mol%	67.5 mol%
Isolation source	Green biofilm	Soil	Soil

271 **Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the
272 relationships between *Nocardioides albertani* sp. nov. CD40127^T and all
273 *Nocardioides* species. The tree was constructed using the neighbour-joining
274 method based on comparison of 1379 nt. Bootstrap values are expressed as
275 percentages of 1,000 replicates; values <50% are not shown. Asterisks indicate
276 that the corresponding branches were also recovered by the maximum-
277 parsimony and maximum-likelihood treeing algorithms. Bar, 0.01 nucleotide
278 substitutions per site. *Terrabacter tumescens* KCTC 9133^T (AF005023) was
279 used as outgroup.

280

281

282

283 **Supplementary Table S1.** Major fatty acid composition of strain CD40127^T and
 284 related type strains.

285 All results from this study were obtained from cells grown on TSA after 3 days at 28°C for strain
 286 CD40127^T, *Nocardioides albus* DSM 43109^T and *Nocardioides luteus* DSM 43366^T. All analysis
 287 were done in triplicate. [†]Summed feature 3 comprises C_{16:1} ω7c and/or iso-C_{15:0} 2-OH.
 288

Fatty Acids	Strain CD40127 ^T	<i>Nocardioides albus</i> DSM 43109 ^T	<i>Nocardioides luteus</i> DSM 43366 ^T
Saturated			
C _{16:0}	0.92	2.89	0.65
C _{17:0}	0.88	1.36	1.76
C _{18:0}	0.63	2.20	0.37
Unsaturated			
C _{17:1} ω6c	1.11	4.46	2.08
C _{17:1} ω8c	1.07	1.50	2.35
C _{18:1} ω9c	0.87	5.82	0.60
Branched			
iso-C _{14:0}	2.85	1.13	1.05
iso-C _{15:0}	1.16	1.18	0.83
iso-C _{16:0}	72.63	58.03	70.21
iso-C _{16:1} H	0.82	0.68	1.34
anteiso-C _{17:0}	3.30	1.11	2.29
iso-C _{18:1}	2.90	1.73	1.34
10-Methyl			
C _{16:0} 10-methyl	1.63	2.58	1.64
C _{17:0} 10-methyl	3.55	4.05	8.62
C _{18:0} 10-methyl	2.55	8.35	1.99
Summed features[†]			
Summed Feature 3	1.02	1.02	0.75

289