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

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

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

A Gram-positive, aerobic, non-spore-forming, rod- or coccoid-shaped, strain CD40127^T, was isolated from a green biofilm covering the wall of the Domitilla Catacombs in Rome, Italy. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CD40127^T belongs to the genus *Nocardioides*, closely related to *Nocardioides luteus* DSM 43366^T and *Nocardioides albus* DSM 43109^T with 98.86% and 98.01% similarity values, respectively. Strain CD40127^T exhibited 16S rRNA gene sequence similarity values below 96.29% with the rest of *Nocardioides* species. The G+C content of the genomic DNA was 69.7 mol%. The predominant fatty acid was iso-$C_{16:0}$ and the major menaquinone was MK-8 (H_4) according to the genus *Nocardioides*. A polyphasic approach using physiological tests, fatty acid profiles, DNA base ratios and DNA-DNA hybridization showed that isolate CD40127^T belongs to a novel species within the genus *Nocardioides*, for which the name *Nocardioides albertani* is proposed. The type strain is CD40127^T (=DSM 25218^T =CECT 8014^T).

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

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

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

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

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

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

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

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

strain CD40127^T occurred in the temperature range of 10-30ºC, with an optimum at 25ºC. Strain CD40127^T grew at NaCl concentrations of 0-10% (w/v) (optimum 0-4%). Table 1 shows other physiological characteristics of strain CD40127^T, as well as numerous phenotypic differences from the phylogenetically closest species of the *Nocardioides* genus. Several physiological and chemotaxonomic differences were noted among strains CD40127^T, *N. albus* DSM 43109^T and *N. luteus* DSM 43366^T. These differences included the growth at 37ºC and spore production. Strain CD40127^T did not grow at 37ºC and either did not produce spore on the tested culture media, while *N. albus* and *N. luteus* grew well at this temperature and produced spores. Other differences were the production of acid from L-rhamnose and the presence or absence of gelatinase, α-mannosidase and trypsin activities. Assimilation of N-acetylglucosamine and maltose differed among the three strains. Further dissimilarities were noticed in fatty acid composition. Although 14-methyl pentadecanoic acid (iso-$C_{16:0}$) is the predominant fatty acid in all three *Nocardioides* species, there were differences in the abundance of fatty acids; $C_{18:0}$ 10-methyl, $C_{18:1}$ω9c and $C_{17:0}$ 10-methyl (Supplementary Table S1). The menaquinone pattern revealed that MK-8 (H_4) was the predominant isoprenoid quinone (76%) according with the genus *Nocardioides*; MK-8 (H_2) was present as a minor component (24%).

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

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

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

Description of *Nocardioides albertani* sp. nov.

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

Cells are Gram-positive, aerobic, non-spore-forming, non-motile and rod-shape 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 28ºC 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 30ºC, optimum at 25ºC. 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-chymotripsin, 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 (H_4).

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.

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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-phosphosphohydrolase and valine arylamidase activities; none of them had oxidase, arginine dihydrolase, α-chymotripsin, 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

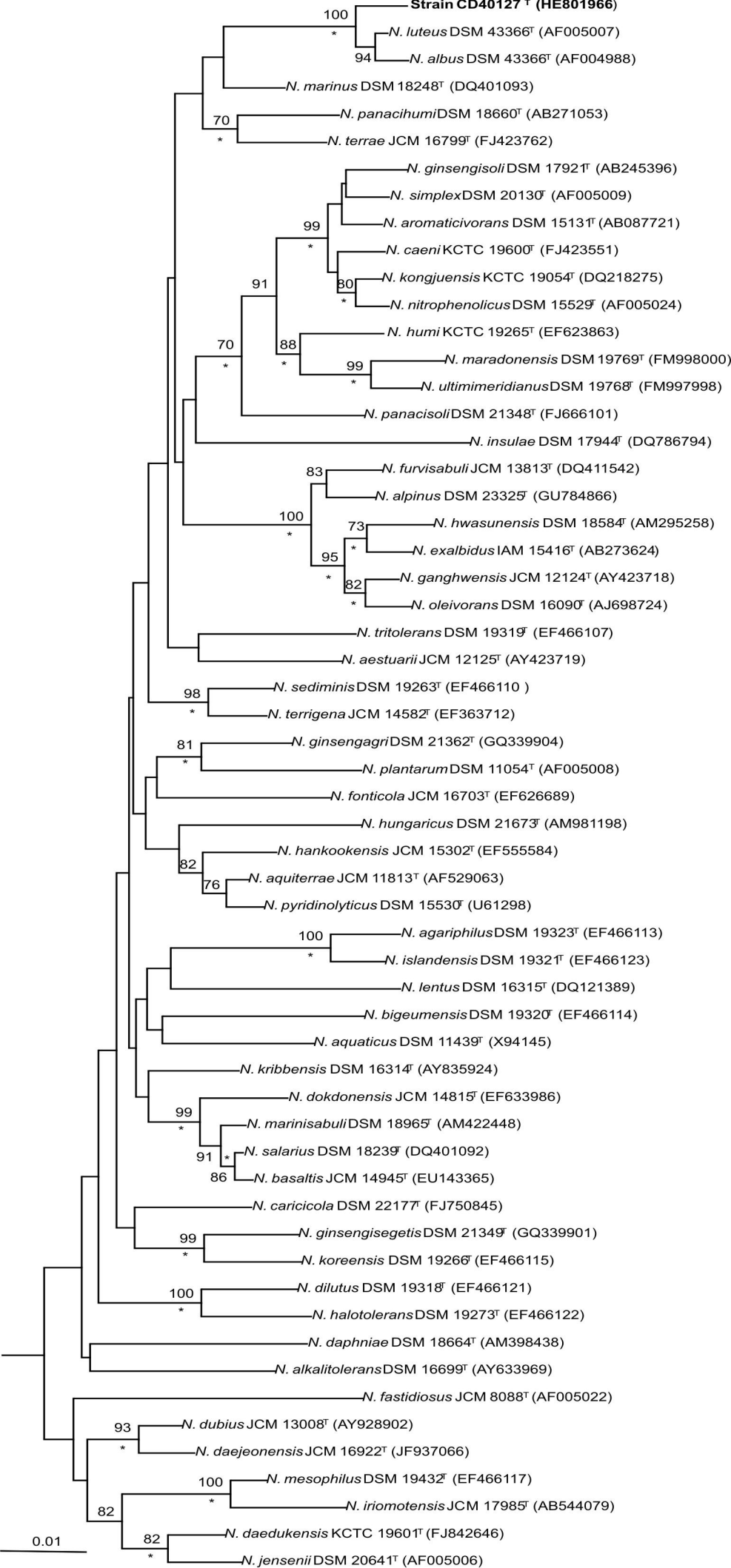
Characteristic	**Strain CD40127^T**	***Nocardioides albus* DSM 43109^T**	***Nocardioides luteus* DSM 43366^T**
Cell morphology	Rods, cocci	Hyphae	Hyphae
Cell length (µm)	1.0-1.6	v	v
Cell width (µm)	0.6-0.8	0.5-1.0	0.5-1.0
Colony colour	cream	cream	Yellow to cream
Growth at 37ºC	-	+	+
Acid produced from 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 $C_{18:1}\omega9c$	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

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

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

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

Fatty Acids	**Strain CD40127^T**	***Nocardioides albus* DSM 43109^T**	***Nocardioides luteus* 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


Strain CD40127 T (HE801966)
100
*
N. luteus DSM 43366T (AF005007)
94
N. albus DSM 43366T (AF004988)
N. marinus DSM 18248T (DQ401093)
N. panacihumi DSM 18660T (AB271053)
70
*
N. terrae JCM 16799T (FJ423762)
N. ginsengisoli DSM 17921T (AB245396)
N. simplex DSM 20130T (AF005009)
99
*
N. aromaticivorans DSM 15131T (AB087721)
N. caeni KCTC 19600T (FJ423551)
91
80
*
N. kongjuensis KCTC 19054T (DQ218275)
N. nitrophenolicus DSM 15529T (AF005024)
N. humi KCTC 19265T (EF623863)
70
*
88
*
99
*
N. maradonensis DSM 19769T (FM998000)
N. ultimimeridianus DSM 19768T (FM997998)
N. panacisoli DSM 21348T (FJ666101)
N. insulae DSM 17944T (DQ786794)
83
N. furvisabuli JCM 13813T (DQ411542)
N. alpinus DSM 23325T (GU784866)
100
*
73
*
N. hwasunensis DSM 18584T (AM295258)
N. exalbidus IAM 15416T (AB273624)
95
*
82
*
N. ganghwensis JCM 12124T (AY423718)
N. oleivorans DSM 16090T (AJ698724)
N. tritolerans DSM 19319T (EF466107)
N. aestuarii JCM 12125T (AY423719)
98
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N. sediminis DSM 19263T (EF466110)
N. terrigena JCM 14582T (EF363712)
81
*
N. ginsengagri DSM 21362T (GQ339904)
N. plantarum DSM 11054T (AF005008)
N. fonticola JCM 16703T (EF626689)
N. hungaricus DSM 21673T (AM981198)
N. hankookensis JCM 15302T (EF555584)
82
76
N. aquiterrae JCM 11813T (AF529063)
N. pyridinolyticus DSM 15530T (U61298)
100
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N. agariphilus DSM 19323T (EF466113)
N. islandensis DSM 19321T (EF466123)
N. lentus DSM 16315T (DQ121389)
N. bigeumensis DSM 19320T (EF466114)
N. aquaticus DSM 11439T (X94145)
N. kribbensis DSM 16314T (AY835924)
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N. marinisabuli DSM 18965T (AM422448)
91
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N. salarius DSM 18239T (DQ401092)
86
N. basaltis JCM 14945T (EU143365)
N. caricicola DSM 22177T (FJ750845)
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N. ginsengisegetis DSM 21349T (GQ339901)
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N. daphniae DSM 18664T (AM398438)
N. alkalitolerans DSM 16699T (AY633969)
N. fastidiosus JCM 8088T (AF005022)
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N. dubius JCM 13008T (AY928902)
N. daejeonensis JCM 16922T (JF937066)
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N. mesophilus DSM 19432T (EF466117)
82
N. iriomotensis JCM 17985T (AB544079)
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