Nocardia grenadensis sp. nov., isolated from sand of the Caribbean Sea

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A Gram-stain-positive, non-spore-forming bacterium (GW5-5797^T) was isolated on soil extract agar from sand collected at a depth of 5 m in the Caribbean Sea near Grenada. 16S rRNA gene sequence analysis and similarity studies showed that strain GW5-5797^T belongs to the genus *Nocardia*, and is most closely related to *Nocardia speluncae* N2-11^T (99.2 % similarity) and *Nocardia jinanensis* 04-5195^T (99.2 %) and more distantly related to *Nocardia rhamnosiphila* 202GMO^T (98.6 %) and other *Nocardia* species. Strain GW5-5797^T could be distinguished from all other recognized *Nocardia* species by sequence similarity values less than 98.5 %. The peptidoglycan diamino acid was *meso*-diaminopimelic acid. Strain GW5-5797^T exhibited a quinone system with the predominant compounds MK-8($H_4\omega$-cyclo) and MK-8(H_2). The polar lipid profile of GW5-5797^T consisted of the major compounds diphosphatidylglycerol, phosphatidylethanolamine and an unidentified glycolipid, moderate amounts of phosphatidylinositol and a phosphatidylinositol mannoside and minor amounts of several lipids including a second phosphatidylinositol mannoside. The polyamine pattern contained the major compound spermine and moderate amounts of spermidine. The major fatty acids were $C_{16:0}$, $C_{18:1}\omega9c$ and 10-methyl $C_{18:0}$. These chemotaxonomic traits are in excellent agreement with those of other *Nocardia* species. The results of DNA–DNA hybridizations and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain GW5-5797^T from the most closely related species, showing 16S rRNA gene sequence similarities >98.5 %. Strain GW5-5797^T therefore merits separate species status, and we propose the name *Nocardia grenadensis* sp. nov., with the type strain GW5-5797^T (=CCUG 60970^T =CIP 110294^T).

The genus *Nocardia* now encompasses more than 70 species of mycolic acid-containing actinomycetes with validly published names. Over the last 3 years, the number of *Nocardia* species has increased to 81, including the recently described species *Nocardia artemisiae* (Zhao *et al.*, 2011), *N. callitridis* (Kaewkla & Franco, 2010), *N. endophytica* (Xing *et al.*, 2011), *N. iowensis* (Lamm *et al.*, 2009), *N. jinanensis* (Sun *et al.*, 2009), *N. mikamii* (Jannat-Khah *et al.*, 2010) and *N. niwae* (Moser *et al.*, 2011). A comprehensive summary of the complex taxonomy of the genus has been given by Goodfellow *et al.* (1999).

During the characterization of organisms isolated from different soils, strain GW5-5797^T was recovered on soil extract agar from 1 g of a sample originating from sand collected at a depth of 5 m in the Caribbean Sea near Grenada, after a 2 h extraction in 10 ml of 0.1 % (v/v) Tween 80 containing 5 mg ampicillin and dilution on mannitol-rifampicin agar [containing (l^{-1}): mannitol, 10 g; yeast extract, 7 g; Casamino acids, 2 g; Bacto peptone, 1 g; NaCl, 1 g; $CaCO_3$, 0.2 g; nystatin, 100 mg; rifampicin, 5 mg] and incubation for 6 weeks at 27 °C. The strain was maintained at 25 °C on DSMZ medium 65 (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium65.pdf) and, on this agar, showed a yellow to orange substrate

Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GW5-5797^T is FR729900.

A supplementary figure is available with the online version of this paper.

mycelium that fragmented easily into irregular rod-shaped cells. Orange–white aerial hyphae were formed.

The strain stained Gram-positive using the procedure of Gerhardt *et al.* (1994), and cell morphology was observed with a Zeiss light microscope at ×1000 using cells grown on medium 65 for 5 days at 25 °C. For PCR amplification of the 16S rRNA gene of strain GW5-5797^T, universal primers 27f and 1492r (Lane, 1991) were used. The 16S rRNA gene sequence (1432 bp) was determined as described by Kämpfer *et al.* (2003). Phylogenetic analysis was performed using the software package MEGA version 4 (Tamura *et al.*, 2007) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura-2 model; Kimura, 1980) were calculated and clustering with the neighbour-joining method (Saitou & Nei, 1987) was performed by using bootstrap values based on 1000 replications (Felsenstein, 1985).

Sequence similarity calculations (on the basis of 1394 nt) after neighbour-joining analysis (Fig. 1) indicated that the closest relatives of strain GW5-5797^T were *Nocardia speluncae* N2-11^T (99.2 %), *N. jinanensis* 04-5195^T (99.2 %) and *Nocardia rhamnosiphila* 202GMO^T (98.6 %). Lower sequence similarities (<98.5 %) were found with other established species of the genus *Nocardia*. A neighbour-joining tree for strain GW5-5797^T and all species of the genus *Nocardia* with validly published names for which nearly full-length 16S rRNA gene sequences are available is shown in Supplementary Fig. S1, available in IJSEM Online. Strain GW5-5797^T still groups with *N. speluncae* and *N. jinanensis*.

Biomass of GW5-5797^T for analysis of the peptidoglycan diamino acid, polar lipids and menaquinones was grown on PYE medium at 25 °C for 72 h (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2). Peptidoglycan diamino acid analysis was carried out as described by Schleifer (1985), and *meso*-diaminopimelic was identified. Analyses of quinones and polar lipids were done as described by Tindall (1990a, b) and Altenburger *et al.* (1996). HPLC analyses of quinones and polyamines were carried out using the procedure of Stolz *et al.* (2007). Strain GW5-5797^T exhibited a quinone system composed of 65 % MK-8($H_4\omega$-cyclo) and 35 % MK-8(H_2). The presence of MK-8($H_4\omega$-cyclo) is a characteristic common to all *Nocardia* species (Goodfellow *et al.*, 1999) analysed so far, including the recently proposed species *N. callitridis* (Kaewkla & Franco, 2010) and *N. mikamii* (Jannat-Khah *et al.*, 2010), but significant amounts of MK-8(H_2) have rarely been reported in *Nocardia* species (Rodríguez-Nava *et al.*, 2004; Xu *et al.*, 2005). The polar lipid profile of GW5-5797^T contained the major compounds diphosphatidylglycerol, phosphatidylethanolamine and an unidentified glycolipid (GL1). Furthermore, moderate amounts of phosphatidylinositol and a phosphatidylinositol mannoside and minor amounts of a second phosphatidylinositol mannoside, two unidentified phospholipids (PL1, PL2) and three unidentified polar lipids (L1–3; not stainable with any of the specific spray reagents molybdenum blue, ninhydrin and α-naphthol) were detected (Fig. 2). The identified lipids in the profile of GW5-5797^T have been shown to be present in several *Nocardia* strains, as well as

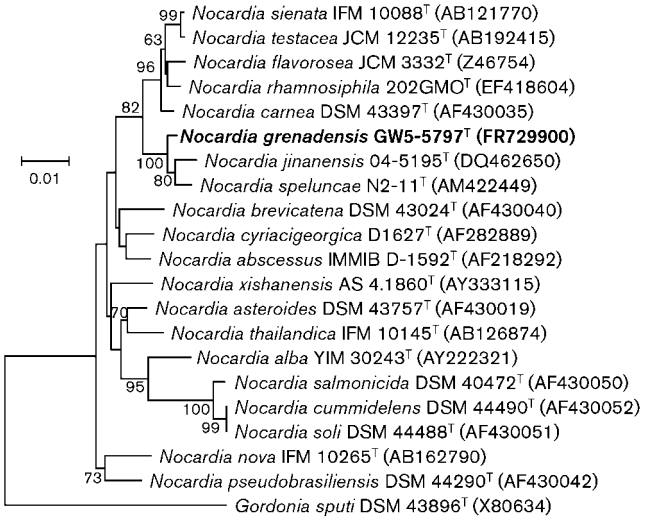

Fig. 1. Neighbour-joining tree reconstructed from the 16S rRNA gene sequences of strain GW5-5797^T and type strains of the genus *Nocardia* (sequences of type strains taken from the EMBL database; accession numbers given in parentheses). Bootstrap values are percentages based on 1000 replications; only values >50 % are shown. Bar, 0.01 substitutions per nucleotide position. An extended version of this tree is available as Supplementary Fig. S1.

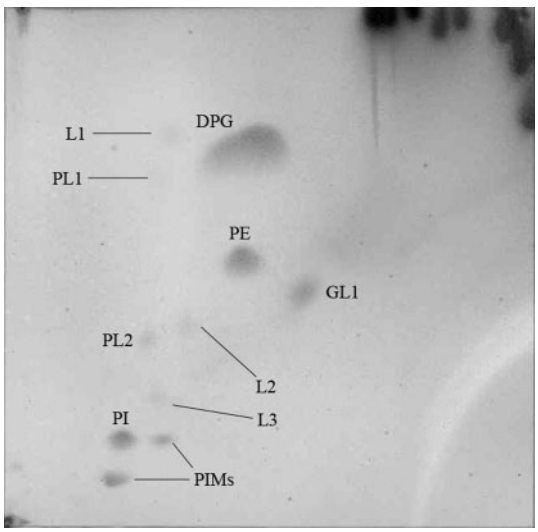

Fig. 2. Total polar lipid profile of strain GW5-5797^T after staining with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIMs, phosphatidylinositol mannosides; GL1, unidentified glycolipid; L1–3, unidentified polar lipids; PL1–2, unidentified phospholipids.

an unidentified glycolipid showing chromatographic behaviour similar to that of GL1 (Minnikin *et al.*, 1977). The presence of the aminolipid phosphatidylethanolamine demonstrates that GW5-5797^T exhibits phospholipid type II (Lechevalier *et al.*, 1977) and thus this polar lipid profile is in accordance with the characteristics of the genus *Nocardia*. Polyamines were extracted and analysed from biomass grown on PYE medium and harvested at the late exponential growth phase as described by Busse & Auling (1988) and Altenburger *et al.* (1997). The polyamine pattern contained the major compound spermine [0.83 μmol (g dry weight)$^{-1}$], moderate amounts of spermidine [0.24 μmol (g dry weight)$^{-1}$] and traces of 1,3-diaminopropane, putrescine and *sym*-homospermidine [<0.03 μmol (g dry weight)$^{-1}$].

Fatty acid analysis was performed according to Kämpfer & Kroppenstedt (1996) using the MIDI Sherlock system version 2.11 (TSBA 4.1). The fatty acid profile of strain GW5-5797^T given in Table 1 was similar to those of closely related species, but also showed some quantitative differences.

Detailed results of the physiological characterization are given in Table 2 and the species description, obtained using methods described previously (Kämpfer *et al.*, 1991). In addition, some tests on the degradation of polymeric substances were performed using standard procedures according to Williams *et al.* (1983). The results of these tests were read after 7 days of incubation at 28 °C.

DNA–DNA hybridization experiments were performed using the method described by Ziemke *et al.* (1998), except that, for nick translation, 2 μg DNA was labelled during a 3 h incubation at 15 °C. Hybridization with labelled DNA from the type strains of related *Nocardia* species gave the following results: *N. speluncae* YEME N2-11^T (18.1 %, reciprocal analysis 14.1 %), *N. jinanensis* DSM 45048^T (34.4, 13.1 %) and *N. rhamnosiphila* DSM 45147^T (15.6, 16.2 %).

On the basis of these results, the novel species *Nocardia grenadensis* sp. nov. is described to accommodate strain GW5-5797^T.

Table 1. Major fatty acids of type strains of species of the genus *Nocardia* grouped into the same cluster on the basis of 16S rRNA gene sequence similarity studies (see Fig. 1)

Strains: 1, GW5-5797^T; 2, *N. speluncae* YEME N2-11^T; 3, *N. jinanensis* DSM 45048^T; 4, *N. rhamnosiphila* DSM 45147^T; 5, *N. carnea* DSM 43397^T. All strains were grown on trypticase soy broth at 28 °C for 7 days prior to fatty acid analysis. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain; *cis* isomers are indicated by the suffix *c*. TBSA, Tuberculostearic acid.

Fatty acid	1	2	3	4	5
Saturated					
$C_{14:0}$	0.9	3.6	1.4	1.0	1.7
$C_{15:0}$	0.6	1.9			1.0
$C_{16:0}$	27.2	25.9	28.6	32.9	25.2
iso-$C_{16:0}$	0.8			1.1	1.3
$C_{17:0}$		2.7	2.9	2.3	1.3
$C_{18:0}$	7.0	11.0	5.6	9.7	7.5
iso-$C_{18:0}$					4.9
$C_{19:0}$	1.0		0.8		
$C_{20:0}$	0.4		1.0		1.6
Unsaturated					
$C_{16:1}\omega9c$	0.6				
$C_{17:1}\omega9c$	0.5				
$C_{17:1}\omega8c$		1.5			
$C_{18:1}\omega9c$	17.2	15.8	20.2	15.9	6.1
$C_{18:1}\omega7c$		1.0			7.2
$C_{20:1}\omega9c$	3.2	2.0	1.1		
Summed features*					
Summed feature 3	11.2	10.2	16.7	10.8	11.2
Summed feature 5		1.8			
Summed feature 6	1.4	2.9	0.8	1.8	3.9
Others					
10-Methyl $C_{17:0}$		1.1			
10-Methyl $C_{18:0}$ (TBSA)	20.8	18.0	19.6	23.0	21.7
Unknown	15.0	0.9	1.0	0.4	5.2

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH. Summed feature 5 contained $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$. Summed feature 6 contained $C_{19:1}\omega11c$ and/or $C_{19:1}\omega9c$.

Description of *Nocardia grenadensis* sp. nov.

Nocardia grenadensis (gre.na.den′sis. N.L. fem. adj. *grenadensis* named after Grenada, from where the type strain was isolated).

Forms a yellow to light-orange vegetative mycelium, fragmenting very rapidly into irregular, rod-shaped elements. Aerial mycelium is yellowish white. Gram-stain-positive and oxidase-positive, showing an oxidative metabolism. Good growth occurs on nutrient agar and medium 65 at 25–30 °C. The characteristic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. The polyamine pattern contains the major compound spermine and moderate amounts of spermidine. Putrescine, 1,3-diaminopropane and *sym*-homospermidine are present in trace amounts. The quinone system is composed of the major menaquinones MK-8($H_4\omega$-cyclo) and MK-8(H_2). The polar lipid profile is composed of the major compounds diphosphatidylglycerol, phosphatidylethanolamine and an unidentified glycolipid, moderate amounts of phosphatidylinositol and a phosphatidylinositol mannoside and minor amounts of a second phosphatidylinositol mannoside, two unidentified phospholipids and three unidentified polar lipids. Major fatty acids are $C_{16:0}$, $C_{18:1}\omega9c$ and 10-methyl $C_{18:0}$. Carbon source utilization and hydrolysis of chromogenic substrates (including differentiating characters) are indicated in Table 1. In addition, urea and allantoin are hydrolysed. Adenine,

Table 2. Physiological properties that distinguish strain GW5-5797^T from the type strains of the most closely related *Nocardia* species

Strains: 1, GW5-5797^T; 2, *N. speluncae* YEME N2-11^T; 3, *N. jinanensis* DSM 45048^T; 4, *N. rhamnosiphila* DSM 45147^T; 5, *N. carnea* DSM 43397^T. All strains were positive for hydrolysis of aesculin, *bis*-*p*-nitrophenyl (pNP) phosphate, pNP phenylphosphonate and L-alanine *p*-nitroanilide (pNA) and utilization of D-glucose, acetate, fumarate, pyruvate and DL-3-hydroxybutyrate. All strains were negative for hydrolysis of pNP β-D-glucuronide, pNP β-D-galactopyranoside, pNP α-D-glucopyranoside, pNP phosphorylcholine, L-alanine pNA, L-glutamyl γ-3-carboxy-pNA and L-proline pNA and utilization of *p*-arbutin, gluconate, L-rhamnose, maltitol, L-sorbose, *cis*- and *trans*-aconitate, adipate, azelate, citrate, glutarate, itaconate, mesaconate, oxoglutarate, β-alanine, L-leucine, L-ornithine, L-tryptophan and 4-hydroxybenzoate. +, Positive, −, negative; (+), weakly positive.

Test	1	2	3	4	5
Hydrolysis of:					
pNP β-D-glucopyranoside	−	−	+	+	−
pNP β-D-xylopyranoside	−	−	−	+	−
pNP phenylphosphonate	−	−	−	+	−
2-Deoxythymidine-5′-pNP phosphate	−	+	−	−	−
Assimilation of:					
N-Acetyl-D-galactosamine	(+)	−	−	−	−
N-Acetyl-D-glucosamine	+	+	−	−	−
L-Arabinose	+	−	−	−	+
Cellobiose	+	−	−	−	−
D-Fructose	+	−	−	−	−
D-Galactose	+	−	−	−	+
D-Mannose	+	−	−	−	−
Maltose	+	−	−	−	−
Melibiose	+	−	−	+	−
D-Ribose	+	+	−	−	−
Sucrose	+	−	−	−	−
Salicin	+	−	−	−	−
Trehalose	+	−	+	+	−
D-Xylose	+	−	−	−	−
Adonitol	+	−	−	−	−
myo-Inositol	+	+	−	−	−
D-Mannitol	+	−	−	−	−
Putrescine	−	+	−	−	−
Propionate	−	+	+	+	+
4-Aminobutyrate	+	−	−	−	+
DL-Lactate	−	−	+	−	−
L-Malate	+	+	−	+	+
Suberate	+	−	−	−	−
L-Alanine	+	+	−	−	−
L-Aspartate	+	−	−	−	−
L-Histidine	+	−	−	−	−
L-Phenylalanine	+	−	−	−	−
L-Proline	+	+	−	−	−
L-Serine	+	−	−	+	−
3-Hydroxybenzoate	+	−	−	+	−
Phenylacetate	+	−	−	+	−

tyrosine, casein and starch (weakly) are degraded, but xylan, hypoxanthine and xanthine are not.

The type strain is GW5-5797^T (=CCUG 60970^T =CIP 110294^T), isolated from sand collected at a depth of 5 m in the Caribbean Sea near Grenada.

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