Nocardioides zeicaulis sp. nov., an endophyte actinobacterium of maize

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A Gram-stain-positive, aerobic organism was isolated as an endophyte from the stem tissue of healthy maize (*Zea mays)* and investigated in detail for its taxonomic position. On the basis of the 16S rRNA gene sequence analysis, strain JM-601^T was shown to be most closely related to *Nocardioides alpinus* (98.3 %), and *Nocardioides ganghwensis* (98.0 %). The 16S rRNA gene sequence similarity to all other species of the genus *Nocardioides* was $\leq$98.0 %. The diagnostic diamino acid of the peptidoglycan was LL-diaminopimelic acid. The major quinone of strain JM-601^T was menaquinone MK-8(H_4). The polar lipid profile revealed the major components diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylcholine and an unidentified phospholipid. The polyamine pattern contained predominantly spermine and moderate amounts of spermidine. In the fatty acid profile, iso-$C_{16:0}$, $C_{17:1}\omega8c$ and 10-methyl $C_{17:0}$ were present in major amounts. All these data support the allocation of the strain to the genus *Nocardioides*. The results of physiological and biochemical characterization allow in addition a phenotypic differentiation of strain JM-601^T from *N. alpinus* and *N. ganghwensis*. Strain JM-601^T represents a novel species of the genus *Nocardioides*, for which we propose the name *Nocardioides zeicaulis* sp. nov., with JM-601^T (=CCM 8654^T=CIP 110980^T) as the type strain.

The genus *Nocardioides* was proposed 40 years ago by Prauser (1976) with *Nocardioides albus* as type species. The genus now contains a number of species which stain Gram-positive and are non-acid-fast, catalase-positive, aerobic and mesophilic nocardioform actinomycetes. They may develop a mycelium that easily fragments into irregular rod- to coccus-like elements. The major chemotaxonomic features are a quinone system with the major menaquinone MK-8(H_4) and LL-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan. The fatty acid profiles are composed of both branched and straight-chain fatty acids (O'Donnell *et al.*, 1982). At the time of writing, the genus *Nocardioides* comprises more than 70 species with validly published names (http://www.bacterio.net/nocardioides.html; Euzéby, 1997). Many of the species have been isolated from a wide variety of sources, like soils, sediments, sand, water, herbage, an oil shale column and glacier cryoconite. Some species of the genus *Nocardioides* were also isolated from plant material including endophytes from different plant species, e.g. *Nocardioides caricicola* from the halophyte *Carex scabrifolia* (Song *et al.*, 2011), *Nocardioides panzhihuensis* from the medicinal plant *Jatropha curcas* (Qin *et al.*, 2012), *Nocardioides perillae* from surface-sterilized roots of *Perilla frutescens* (Du *et al.*, 2013), *Nocardioides endophyticus* and *Nocardioides conyzicola* from surface sterilized roots of mugwort (*Artemisia princeps*) and horse-weed (*Conyza canadensis*) (Han *et al.*, 2013), and *Nocardioides zeae* from *Zea mays* (Glaeser *et al.*, 2014).

Strain JM-601^T was isolated as an endophyte of maize. The strain produced single cells forming small yellow colonies (<0.5 mm) with a smooth surface after 48 h at 28 °C on tryptone soy agar (TSA; Oxoid). Morphological features were recorded with cells grown on TSA at 28 °C by phase-contrast microscopy. During exponential growth, irregularly shaped cells of strain JM-601^T were observed, 0.9–1.5 μm wide and 1.5–2.5 μm sometimes 3–4 μm long, which showed no motility. Cells stained weakly Gram-positive (analysed as described by Gerhardt *et al.*, 1994)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JM-601^T is KU201963.

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and were negative for cytochome oxidase, determined by using an oxidase test (Merck). Endospores could not be observed. Temperature-dependent growth was investigated after growth on TSA at 4, 15, 25, 28, 32, 37 and 42 °C. Salinity- and pH-dependent growth was analysed in tryptic soy broth (Difco) either supplemented with 1 to 10 % (w/v) NaCl (increasing in 1 % increments) or adjusted to pH values between pH 4 and 10 (increasing in 0.5 pH units by the addition of HCl or NaOH); both were cultured at 28 °C.

The nearly full-length 16S rRNA gene of strain JM-601^T was PCR-amplified and sequenced with primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AC GGCTACCTTGTTACGACTT-3′; Lane, 1991). The most closely related type strains were determined by BLAST analysis against the 16S rRNA gene sequence database in EzTaxon-e (Kim *et al.*, 2012). Phylogenetic trees were calculated using ARB release 5.2 (Ludwig *et al.*, 2004) and the latest version (release LTPs123, July 2015) of the 'All-Species Living Tree' Project (LTP) (Yarza *et al.*, 2008). The online alignment tool SINA (v1.2.9; Pruesse *et al.*, 2012) was used to align the sequence of strain JM-601^T and sequences of type strains not included in the LTP database according to the SILVA seed alignment (http://www.arb-silva.de; Pruesse *et al.*, 2007). Aligned sequences were implemented into the database. The alignment including all type strains of the family *Nocardioidaceae* was corrected manually before phylogenetic trees were calculated. A maximum-likelihood tree was calculated with RAxML v7.04 (Stamatakis, 2006) using GTR-GAMMA and rapid bootstrap analysis and a maximum-parsimony tree using DNAPARS v 3.6 (Felsenstein, 2005). Phylogenetic trees were based on 1000 resamplings (bootstrap analysis; Felsenstein, 1985) and 16S rRNA gene sequences between gene termini 66 and 1435 (numbering according to Brosius *et al.*, 1978).

The 16S rRNA gene sequence of strain JM-601^T is a continuous stretch of 1425 nt spanning gene termini 66 to 1435 (*Escherichia coli* numbering; Brosius *et al.*, 1978). The most closely related type strains obtained by the EzTaxon BLAST analysis were *Nocardioides alpinus* Cr7-14^T (GU784866) and *Nocardioides ganghwensis* JC2055^T (AY423718) with 98.3 and 98.0 % 16S rRNA gene sequence similarity, followed by *Nocardioides furvisabuli* SBS-26^T (DQ411542), *Nocardioides oleivorans* DSM 16090^T (AJ698724), *Nocardioides exalbidus* RC825^T (AB273624) and *Nocardioides hwasunensis* HFW-21^T (AM295258) with 97.9, 97.8, 97.5 and 97.3 %, respectively. Sequence similarities of strain JM-601^T to all other *Nocardioides* type strains were below 97 %. Phylogenetic trees showed, independent of the treeing methods applied, the placement of strain JM-601^T into the genus *Nocardioides* within a distinct cluster (bootstrap support >70 %) including the above-mentioned type strains and *Nocardioides glacieisoli* HLT3-15^T (JQ673418) (Fig. 1).

Cellular biomass subjected to analyses of polyamines, diamino acid, quinones and polar lipids was grown in PYE broth (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) at 28 °C. For polyamine analysis, biomass was harvested at the late exponential growth phase as recommended by Busse & Auling (1988) whereas biomasses used for extraction of diamino acids, quinones and polar lipids were harvested at the stationary growth phase. Polyamines were extracted as reported by Busse & Auling (1988) and Altenburger *et al.* (1997) and analysed using HPLC conditions described by Busse *et al.* (1997). Identification of the isomer of diaminopimelic acid was carried out according to the protocol of Schumann (2011). Quinones and polar lipids were extracted and analysed as described by Tindall (1990a, b) and Altenburger *et al.* (1996). The HPLC apparatus used was described by Stolz *et al.* (2007). The diagnostic diamino acid of the peptidoglycan was LL-diaminopimelic acid. Strain JM-601^T showed a quinone system which contained 93.6 % menaquinone MK-8(H_4), 0.3 % MK-8(H_2) and 6.1 % MK-8. The polar lipid profile (Fig. S2) consisted of the major lipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and one unidentified phospholipid (PL2), and moderate amounts of phosphatidylcholine, one unidentified phospholipid (PL1) and five lipids (L1–5) not containing a sugar residue, an amino group or a phosphate group. Again, the quite rarely detected phospholipid phosphatidylcholine was detected in this strain, similar to *N. zeae* (Glaeser *et al.*, 2014). The polyamine pattern contained [in μmol (g dry weight)$^{-1}$]: 3.02 spermidine, 9.37 spermine, 0.54 putrescine, and 0.88 tyramine. The detection of LL-diaminopimelic acid in the peptidoglycan as well as the quinone system consisting predominantly of menaquinone MK-8(H_4) is in accordance with the description of the genus *Nocardioides* (O'Donnell *et al.*, 1982). The polar lipid profile of strain JM-601^T showed a high degree of similarity with those of other species of the genus *Nocardioides* in respect to presence of phospholipids, including *N. albus*, *N. luteus*, *Nocardioides simplex* (O'Donnell *et al.*, 1982; Collins *et al.*, 1983), *Nocardioides dubius* (Yoon *et al.*, 2005) and *N. zeae* (Glaeser *et al.*, 2014). The polyamine pattern of strain JM-601^T with the major compounds spermidine and spermine is similar to that of *N. zeae* (Glaeser *et al.*, 2014) but it clearly differentiates strain JM-601^T from *N. albus*, *N. luteus*, *Nocardioides jensenii* and *Nocardioides plantarum*, which were reported to contain polyamine patterns with the major compounds putrescine and cadaverine or cadaverine and spermine (Busse & Schumann, 1999).

Fatty acids analysis of cells, grown in R2A broth at 28 °C, was conducted as described by Kämpfer & Kroppenstedt (1996). The fatty acid profile comprised mainly iso- and anteiso-branched fatty acids and was similar to those of the most closely related species (Table 1). However, strain JM-601^T produced a relatively high amount of 10-methyl-$C_{17:0}$ compared with related species of the genus *Nocardioides* (Table 1).

The results of the physiological characterization, performed using methods described previously (Kämpfer, 1990;

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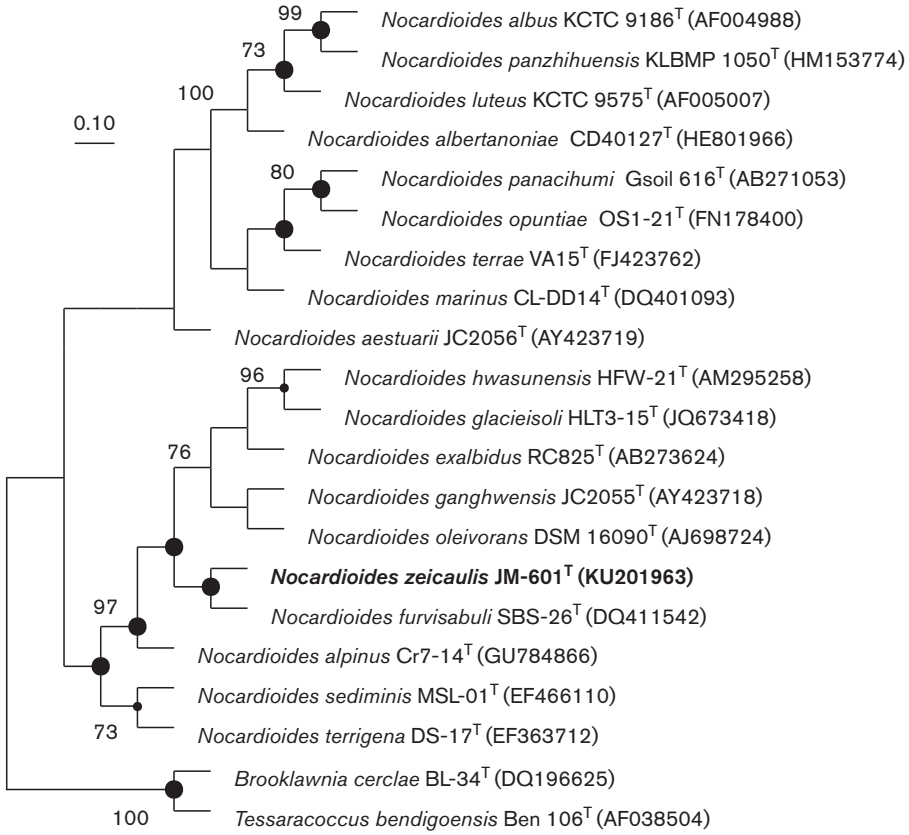

Fig. 1. Reduced maximum-likelihood tree showing the phylogenetic position of strain JM-601^T among the most closely related *Nocardioides* type strains (including that of the type species, *N. albus*). The phylogenetic tree is a reduced tree of the original tree calculated (see Fig. S1 available in the online Supplementary Material) by the inclusion of all species of the *Nocardioidaceae* and the type strains of *Brooklawnia cerclae* and *Tessaracoccus bendigoensis* as outgroups. The tree was generated in ARB using RAxML (GTR-GAMMA, Rapid Bootstrap analysis) and based on nucleotide sequences among 16S rRNA gene sequence positions 66 to 1435 (according to *E. coli* numbering). Bootstrap values ≥70 % are given at nodes in the tree. Circles at nodes represent nodes that also occurred in the maximum-parsimony tree calculated in parallel; large circles represent nodes that were also supported by high bootstrap values in the maximum-parsimony tree. Bar, 0.1 substitutions per nucleotide position. *Nocardioides* type strains which were not directly related to JM-601^T were removed from the tree after tree reconstruction without changing the overall tree topology.

Kämpfer *et al.*, 1991), are given in Table 2 and in the species description. Strain JM-601^T was able to utilize many sugars or sugar-related compounds. A distinct physiological biochemical profile allowed differentiation from the type strains of *N. alpinus* and *N. ganghwensis*. DNA–DNA hybridization experiments were not performed because the 16S rRNA gene sequence similarities were <98.4 %. This level of sequence similarity is below the value for which the maximum probability of error is 0.25 % (Meier-Kolthoff *et al.*, 2013). Kim *et al.* (2014) recently recommended a threshold of 98.65 %.

From the results of the phylogenetic and chemotaxonomic analyses it is clear that strain JM-601^T warrants novel species status, which is by now allocated to the genus *Nocardioides*. For this species we propose the name *Nocardioides zeicaulis*.

Description of *Nocardioides zeicaulis* sp. nov.

Nocardioides zeicaulis (ze.i.cau′lis. L. fem. n. *zea* spelt, also the genus name of corn – *Zea mays*; L. masc. n. *caulis* stem, stalk; N.L. gen. n. *zeicaulis* of the stem of *Zea mays*).

Cells are Gram-stain-positive, strictly aerobic, irregular rods (0.9–1.5 × 1.5–2-5 μm) and non-motile. Colonies grown on tryptone soy agar are circular, convex and yellowish. Optimal temperature for growth is 28 °C; growth occurs between 10 and 37 °C but not at 5 or 50 °C on TSA. Optimal pH for growth is pH 7.0; growth occurs between pH 5.5 and 8.0. Growth occurs in the presence of 1 to 3 % NaCl but not at concentrations above in tryptic soy broth. Test for catalase is positive; oxidase activity is negative. No acid formation from sugars can be observed from D-glucose, D-xylose, lactose, sucrose, D-mannitol,

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Table 1. Cellular fatty acid contents (percentages) of strain JM-601^T as compared with the type strains of phylogenetically closely related species of the genus *Nocardioides*

Strains: 1, JM-601^T; 2, *N. alpinus* Cr7-14^T; 3, *N. ganghwensis* KACC 20321^T. Data for taxa 1–3 from this study. Data for taxa 2 and 3 in parentheses are from Zhang *et al.* (2012). All strains were incubated on R2A agar plates at 25 °C for 48 h prior to fatty acid analysis. ND, Not detected.

Fatty acid	1	2	3
Straight chain fatty acids			
$C_{14:0}$	ND	ND (0.1)	ND (0.2)
$C_{16:0}$	ND	1.7 (2.0)	1.7 (1.4)
$C_{17:0}$	5.4	6.6 (3.5)	4.6 (1.6)
$C_{18:0}$	ND	(ND)	2.3 (0.4)
Branched fatty acids			
iso-$C_{14:0}$	1.9	ND (1.0)	ND (1.9)
iso-$C_{15:0}$	2.7	1.0 (1.4)	3.2 (5.2)
anteiso-$C_{15:0}$	ND	ND (0.1)	ND (0.2)
iso-$C_{16:0}$	38.8	42.2 (32.4)	28.0 (30.1)
iso-$C_{16:1}$ H	2.5	4.0 (3.5)	1.8 (3.1)
iso-$C_{17:0}$	5.3	1.1 (1.1)	6.0 (4.7)
anteiso-$C_{17:0}$	ND	ND (0.3)	ND (0.4)
iso-$C_{17:1}\omega9c$	3.4	ND (ND)	2.1 (ND)
iso-$C_{18:0}$	2.5	ND (0.6)	2.1 (1.1)
iso-$C_{18:1}$ H	ND	ND (0.8)	ND (0.6)
Unsaturated fatty acids			
$C_{15:1}\omega6c$	ND	ND (0.5)	ND (0.3)
$C_{17:1}\omega6c$	ND	1.3 (2.3)	ND (1.9)
$C_{17:1}\omega8c$	19.6	38.4 (39.5)	19.8 (23.1)
$C_{18:1}\omega7c$	ND	ND (0.6)	1.4 (0.9)
$C_{18:1}\omega9c$	5.6	4.5 (3.3)	19.0 (11.5)
10-Methyl fatty acids			
10-Methyl $C_{16:0}$	ND	(0.8)	3.0 (3.9)
10-Methyl $C_{17:0}$	12.3	(1.7)	2.3 (2.5)
10-Methyl $C_{18:0}$ (TBSA)	ND	ND (ND)	ND (ND)
10-Methyl $C_{19:0}$	ND	ND (ND)	ND (ND)
Hydroxy fatty acids			
$C_{17:0}$ 3-OH	ND	ND (0.8)	ND (0.3)
Summed features*			
3	ND	2.6 (2.5)	1.9 (3.3)
6	ND	ND (0.6)	ND (0.7)

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$. Summed feature 6 comprises $C_{19:1}\omega9c$ and/or $C_{19:1}\omega11c$.

Table 2. Phenotypic characteristics that differentiate strain JM-601^T from the type strains of phylogenetically closely related species of the genus *Nocardioides*

Strains: 1, JM-601^T; 2, *N. alpinus* Cr7-14^T; 3, *N. ganghwensis* KACC 20321^T. Phenotypic comparisons for all strains were done at 25 °C. Data for taxa 1−3 from this study. All strains are positive for catalase, nitrate reduction, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, cystine arylamidase, valine arylamidase, α-glucosidase, protease and lipase esterase. All strains are negative for: growth under anaerobic conditions; cytochrome oxidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, indole production, arginine dihydrolase, urease, H_2S production, lysine dihydrolase, ornithine dihydrolase and tryptophan deaminase; assimilation of trisodium citrate, phenylacetic acid, capric acid and adipic acid; and fermentation of glucose, mannitol, sucrose, inositol, sorbitol, rhamnose, melibiose, amygdalin, L-arabinose, ribose, xylose, maltose, lactose and glycogen. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3
Isolation source	Endophyte, *Zea mays*	Alpine glacier cryoconite	Tidal flat sediment
Growth on R2A agar at/with:			
5 °C	−	+	−
30 °C	+	−	+
37 °C	+	−	+
pH 6	+	−	−
5 % (w/v) NaCl	−	−	W
Growth on TSA	−	−	−
Enzyme activities			
Gelatinase	−	−	+
Amylase	+	−	+
α-Galactosidase	+	−	−
β-Galactosidase	W	−	+
Pyrazinaminidase	−	−	−
Assimilation of (API 20NE):			
Glucose	+	+	−
L-Arabinose	+	+	+
D-Mannose	+	W	−
N-Acetylglucosamine	−	−	W
Maltose	+	+	+
Malic acid	−	W	−

dulcitol, salicin, D-adonitol, *myo*-inositol, D-sorbitol, L-arabinose, raffinose, L-rhamnose, maltose, trehalose, cellobiose, erythritol, melibiose or D-arabitol. Several sugar compounds are utilized: D-fructose, cellobiose, D-galactose, D-gluconate, D-glucose, maltose, D-mannose, L-rhamnose, sucrose, melibiose, trehalose and D-xylose. Arbutin, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, ribose, D-adonitol, *myo*-inositol, D-sorbitol and salicin are not utilized. Major fatty acids are iso-$C_{16:0}$, $C_{17:1}\omega8c$ and 10-methyl-$C_{17:0}$ in major amounts and $C_{17:0}$, iso-$C_{17:0}$ and $C_{18:1}\omega9c$ in minor amounts. The diagnostic diamino acid of the peptidoglycan is LL-diaminopimelic acid. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and an unidentified phospholipid (PL2). Moderate amounts of phosphatidylcholine, an unidentified phospholipid (PL1) and five unidentified polar lipids not containing a sugar, phosphate or amino residue are present as well. The major quinone is menaquinone MK-8(H_4). In the polyamine pattern, spermidine and spermine predominate.

The type strain, JM-601^T (=CCM 8654^T=CIP 110980^T), was isolated as an endophyte of maize in July 1990 from

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stem tissue of healthy 10-week-old, field-grown maize (*Zea mays*, cultivar 'Sweet Belle') at the Plant Breeding Unit facility, E.V. Smith Research Center, Tallassee, Alabama, USA.

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