Nocardioides endophyticus sp. nov. and *Nocardioides conyzicola* sp. nov., isolated from herbaceous plant roots

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Two Gram-stain-positive, non-motile, non-spore-forming, rod-shaped actinobacterial strains were isolated from the surface-sterilized roots of mugwort (*Artemisia princeps*) and horse-weed (*Conyza canadensis*), and subjected to taxonomic characterization. 16S rRNA gene sequence analysis indicated that the isolates, designated MWE 3-5^T and HWE 2-02^T, should be placed in the genus *Nocardioides* of the family *Nocardioidaceae*. The strains were closely related to *Nocardioides hankookensis* DS-30^T, which exhibited 16S rRNA gene sequence similarity values of 97.99 and 99.09 % with strains MWE 3-5^T and HWE 2-02^T, respectively. The genome relatedness of *N. hankookensis* DS-30^T with strain MWE 3-5^T was 35.8 %, and that with strain HWE 2-02^T was 36.4 %, whereas that between the two isolates was 43.2 %. Strains MWE 3-5^T and HWE 2-02^T possessed MK-8(H_4) as the major isoprenoid quinone, and LL-diaminopimelic acid in the cell-wall peptidoglycan. The main fatty acids were iso-$C_{16:0}$, iso-$C_{17:0}$ and $C_{18:1}\omega9c$ for strain MWE 3-5^T and iso-$C_{16:0}$, 10-methyl $C_{18:0}$ and $C_{18:1}\omega9c$ for strain HWE 2-02^T. Based on phenotypic, genotypic and phylogenetic studies, the following two novel species are proposed: *Nocardioides endophyticus* sp. nov. (type strain, MWE 3-5^T=KCTC 29122^T=JCM 18532^T) and *Nocardioides conyzicola* sp. nov. (type strain, HWE 2-02^T=KCTC 29121^T=JCM 18531^T).

The genus *Nocardioides* was proposed by Prauser (1976) with a single species, *Nocardioides albus*, and at the time of writing the genus encompasses 63 species (http://www.bacterio.net). Five of these species have been discovered from plants and rhizosphere: *Nocardioides ultimimeridianus* and *Nocardioides maradonensis* from the rhizosphere soil of *Peucedanum japonicum* (Lee *et al.*, 2011), *Nocardioides caricicola* from the root of *Carex scabrifolia* (Song *et al.*, 2011), *Nocardioides panzhihuaensis* from the stem of *Jatropha curcas* (Qin *et al.*, 2012) and *Nocardioides perillae* from the root of *Perilla frutescens* (Du *et al.*, 2013). Other recent studies also suggest members of the genus *Nocardioides* are commonly found as endophytic bacteria (López-López *et al.*, 2010; Kaewkla & Franco, 2013). In this study, two endophytic bacterial strains designated MWE 3-5^T and HWE 2-02^T were isolated from surface-sterilized plant roots. Based on this taxonomic study using a polyphasic approach, each of these two strains is proposed to represent novel species of the genus *Nocardioides*.

During the investigation of endophytic bacterial diversity in Korean native plants, mugwort (*Artemisia princeps*) and horse-weed (*Conyza canadensis*) were sampled from Daejeon area. Plant roots were separated from soil and washed and sterilized following a previously described procedure (Park *et al.*, 2005), and additionally treated with 2.5 % $Na_2S_2O_3$ for 5 min before rinsing in sterile distilled water. The surface-sterilized roots were pulverized in a ceramic mortar. Serial dilutions were spread on R2A agar (Difco). Isolates were obtained after incubation of the inoculated plates at 30 °C for 7 days. Isolates were cultivated on R2A agar or in R2A broth at 30 °C for 5 days, and also maintained in 20 % glycerol at −70 °C.

Abbreviations: PG, phosphatidylglycerol; PIM, phosphatidylinositol mannoside.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MWE 3-5^T and HWE 2-02^T are KC878444 and KC878445, respectively.

Two supplementary figures are available with the online version of this paper.

Genomic DNA was extracted using a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primers 27F and 1492R, and the purified PCR products were sequenced using the service of Macrogen. The resultant 16S rRNA gene sequences were aligned together with corresponding sequences of representative species of the genus *Nocardioides* using the PHYDIT program version 3.1. Evolutionary distances were calculated using the Jukes and Cantor model, and phylogenetic trees were inferred based on the neighbour-joining method using the PHYLIP software package (Felsenstein, 1985). Bootstrap analysis was carried out using 1000 resampled datasets, and maximum-likelihood and maximum-parsimony algorithms were also used to test the topology of the neighbour-joining tree. In the phylogenetic tree, strains MWE 3-5^T and HWE 2-02^T were positioned within the genus *Nocardioides* and clustered with *Nocardioides hankookensis* DS-30^T, as the 16S rRNA gene sequence similarity values between the isolates and the latter were 97.99 and 99.09 %, respectively (Fig. 1). The 16S rRNA gene sequence similarity value between strain MWE 3-5^T and strain HWE 2-02^T was 98.78 %, and those between the two isolates and the other species of the genus *Nocardioides* were less than 98.10 %. The isolates were not related to other known plant-associated species with the exception of *N. caricicola* YC6903^T, which shared 16S rRNA gene sequence similarities of 96.20 and 96.34 % with the isolates (Fig. 1).

The Gram reaction was performed by the standard Gram-staining method. Catalase activity was investigated by bubble production in 3 % (v/v) hydrogen peroxide and oxidase activity was determined using 1 % (w/v) tetramethyl-*p*-phenylendiamine. Tests for degradation of casein (5 % skimmed milk) and starch were evaluated after 7 days of incubation at 30 °C. Biochemical tests were performed using the API 20NE, API 50CH and API ZYM systems (bioMérieux). Growth was examined after 7 days of incubation at various temperatures (4–42 °C) on R2A, and at pH 4–10 in 1/10 diluted R2A broth. Three different buffers (0.5 M final concentration) were used to adjust the pH of R2A broth. Sodium acetate buffer was used for pH 4–6, potassium phosphate buffer was used for pH 7–9, and sodium carbonate buffer was used for pH 10. Tolerance of NaCl was determined in R2A broth supplemented with 0–5 % (w/v) NaCl. For pH and NaCl tolerance studies, cells were incubated in the shaker, and growth was determined spectrophotometrically at OD_{600} after 7 days.

Good growth occurred at temperatures ranging from 25 to 30 °C and at pH 6–7. Colonies were whitish, circular to slightly irregular with entire margins, convex and smooth. Cells were rod-shaped (Fig. S1, available in IJSEM Online), Gram-stain-positive, aerobic, non-spore-forming, oxidase- and catalase-positive. The phenotypic characteristics of strains MWE 3-5^T, HWE 2-2^T and *N. hankookensis* DS-30^T are summarized in the species descriptions and Table 1.

The cellular fatty acid profiles of strain MWE 3-5^T, HWE 2-02^T and *N. hankookensis* DS-30^T were determined using cells grown on R2A for 3 days at 30 °C. Cellular fatty acids were extracted according to the Sherlock Microbial Identification System (MIDI) protocol and analysed by gas chromatography (7890; Hewlett Packard) using the Microbial Identification software package with the Sherlock system MIDI 6.1 and RTSBA6 database. Respiratory quinones were extracted with chloroform/methanol and purified using Sep-Pak Vac silica cartridges and analysed by HPLC as described previously (Komagata & Suzuki, 1987). The isomer of the diamino acid in the cell-wall peptidoglycan was also determined by using TLC as described by Komagata & Suzuki (1987). Polar lipids were extracted from freeze-dried cells and separated by two-dimensional silica gel thin-layer chromatography (Merck). The first direction was developed in chloroform/methanol/water (65 : 25 : 3.8, by vol.), and the second in chloroform/methanol/acetic acid/water (40 : 7.5 : 6 : 1.8, by vol.). Total lipids and specific functional groups were detected using molybdophosphoric acid (for total lipids), molybdenum blue spray reagent (for phosphate), ninhydrin (for free amino groups), periodate–Schiff (for α-glycols) and α-naphthol reagent (for sugars).

The fatty acid profiles of the two strains and reference strain are shown in Table 2. The main fatty acids of strain MWE 3-5^T were iso-$C_{16:0}$ (56.2 %), iso-$C_{17:0}$ (9.1 %), $C_{18:1}\omega 9c$ (7.5 %) and iso-$C_{15:0}$ (6.9 %), and those of strain HWE 2-02^T were iso-$C_{16:0}$ (61.5 %), 10-methyl $C_{18:0}$ (6.9 %) and $C_{18:1}\omega 9c$ (5.9 %). The major respiratory quinone and the diagnostic diamino acid in the cell-wall peptidoglycan of both strains was MK-8(H_4) and LL-2,6-diaminopimelic acid, respectively. The polar lipid profiles of strains MWE 3-5^T, HWE 2-02^T and *N. hankookensis* DS-30^T are shown in Fig. S2. The polar lipids of these strains consisted mainly of phosphatidylinositol mannoside (PIM), phosphatidylglycerol (PG), unidentified phospholipids and unidentified polar lipids. The presence of PIMs has been reported for other species of the genus *Nocardioides* (Evtushenko *et al.*, 2012), but it is notable that PIMs were the major components for the three species analysed in this study.

The DNA base compositions were determined through thermal denaturation fluorimetric method (Gonzalez & Saiz-Jimenez, 2002) using SYBR Green 1 (SG 1; Invitrogen) and CFX Connect Real-Time PCR Detection System (Bio-Rad). Genomic DNA from *Acinetobacter baumannii* KACC 12454^T (39.10 mol% DNA G+C content), *Bacillus subtilis* subsp. *spizizenii* KACC 14741 (43.80 mol%), *Corynebacterium glutamicum* KACC 10784 (53.81 mol%), *Aeromonas hydrophila* ATCC 7966^T (61.50 mol%) and *Micrococcus luteus* KACC 10488^T (73.00 mol%), of which the genomes have been completely sequenced, were used as calibration references. Thermal denaturation was performed with 50 µl reaction mixture containing 0.1 × standard saline citrate, SYBR Green I at a dilution of 1 : 100 000 and approximately 200 ng DNA from the isolates and the calibration reference strains. The thermal cycler conditions consisted of a ramp from 45 to 99 °C at 1.2 °C min^{-1}. Fluorescent DNA melting curves were generated in triplicates. The DNA G+C contents for the isolates were calculated using a linear regression

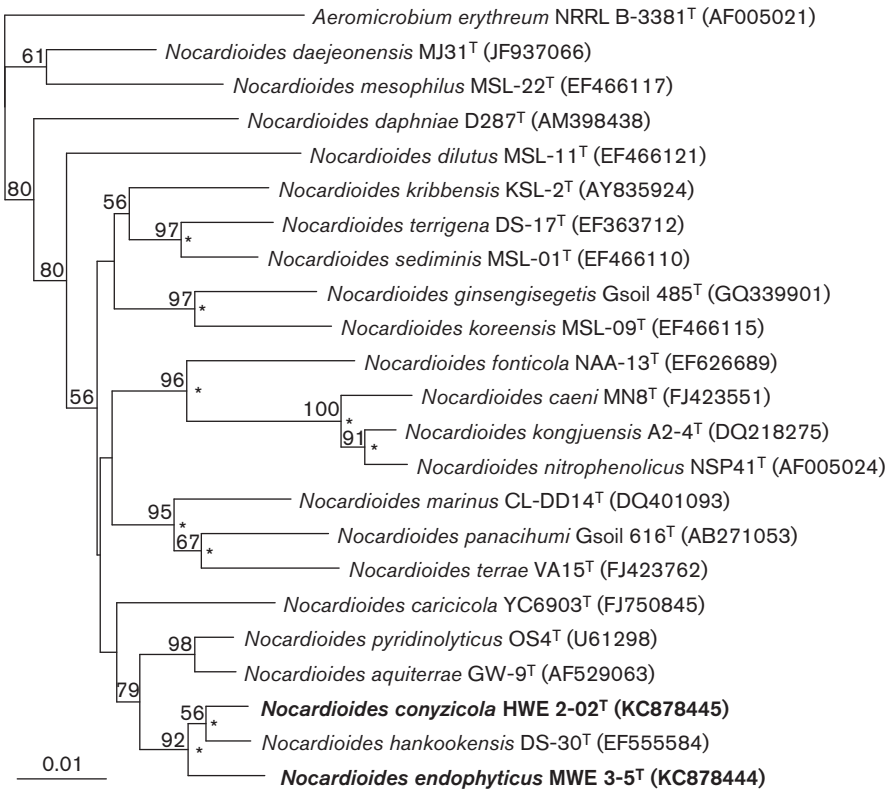

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships among isolates MWE 3-5^T, HWE 2-02^T and other related strains. The value above each branch indicates the percentage levels of bootstrap support (>50 %) for the branch point based on 1000 resamplings. Asterisks indicate branches that were also recovered in the maximum-parsimony and maximum-likelihood trees. Bar, 0.01 changes per nucleotide position.

analysis of melting temperatures against calibration reference strains. The DNA G+C content of strain MWE 3-5^T was 68.51 mol% and of strain HWE 2-02^T was 68.47 mol%, which are within the range reported for the genus *Nocardioides* (Evtushenko *et al.*, 2012). DNA–DNA hybridization was performed fluorometrically using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and microdilution wells. The DNA–DNA relatedness value of *N. hankookensis* DS-30^T with strain MWE 3-5^T was 35.8 %, the value with strain HWE 2-02^T was 36.4 %, and that between the two novel isolates was 43.2 %.

The combination of phenotypic and genotypic characteristics clearly differentiated strains MWE 3-5^T and HWE 2-02^T from previously described species of the genus *Nocardioides* and also from each other, and accordingly strains MWE 3-5^T and HWE 2-02^T represent two novel species of the genus *Nocardioides*, for which the names *Nocardioides endophyticus* sp. nov. and *Nocardioides conyzicola* sp. nov. respectively, are proposed.

Description of *Nocardioides endophyticus* sp. nov.

Nocardioides endophyticus (en.do.phy′ti.cus. Gr. pref. *endo*- within; Gr. neutr. n. *phyton* plant; N.L. adj. *endophyticus* within plant, pertaining to the isolation of the type strain from plant root).

Cells are aerobic, non-spore-forming, non-motile, short and irregular rods (0.3–0.4 × 0.5–1.6 μm). Colonies are whitish, circular to slightly irregular with entire margins, convex, smooth, and glistening. Growth occurs at 10 and 37 °C (optimum 30 °C), but not at 4 or 42 °C. The optimum pH for growth is pH 6.0. Growth occurs in the presence of 0–1.0 % (w/v) NaCl, but not with ⩾2.0 %. Catalase- and oxidase-positive. Nitrate is reduced to nitrite. Data on carbon assimilation (API 20NE) and enzyme activities (API ZYM) are given in Table 1. The diagnostic diamino acid in the cell-wall peptidoglycan is LL-2,6-diaminopimelic acid. The predominant menaquinone is MK-8(H_4) and the major fatty acids are iso-$C_{16:0}$ and iso-$C_{17:0}$. The polar lipids consist of PIM, PG, unidentified phospholipids and unidentified polar lipids.

The type strain is MWE 3-5^T (=KCTC 29122^T=JCM 18532^T), which was isolated from the surface-sterilized root of *Artemisisa princeps*. The genomic DNA G+C content of the type strain is 68.51 mol%.

Table 1. Differential characteristics between strains MWE 3-5^T, HWE 2-02^T and *N. hankookensis* DS-30^T

Strains: 1, MWE 3-5^T; 2, HWE 2-02^T; 3, *N. hankookensis* DS-30^T. All strains were positive for oxidase and catalase activity, nitrate reduction, and hydrolysis of casein and starch, but negative for motility. For API 20NE test, all strains were positive for aesculin hydrolysis and assimilation of *N*-acetylglucosamine. For API ZYM test, all strains were positive for alkaline phosphate, esterase lipase (C8), leucine arylamidase, acid phophatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase activities. For API 50CH test, all strains were negative for methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, melibiose, inulin, melezitose, raffinose, glycogen, xylitol, turanose, D-lyxose and D-tagatose. +, Positve; −, negative; W, weakly positive.

Characteristic	1	2	3
Isolation source	Plant root	Plant root	Soil
Optimal growth temperature (°C)	30	25–30	25
Hydrolysis of:			
Gelatin	+	+	−
Assimilation of (API 20NE):			
p-Nitrophenyl β-D-galactopyranoside	−	+	+
Glucose	+	−	+
Arabinose	+	−	+
Mannitol	+	−	+
Maltose	+	−	−
Gluconate	−	+	W
Adipate	−	W	+
Malate	+	−	+
Carbon source utilization (API 50CH)			
Erythritol	+	−	−
Methyl β-D-xylopyranoside	+	−	+
D-Sorbose	+	+	−
L-Rhamnose	+	+	−
Dulcitol	+	+	−
Inositol	+	+	−
D-Mannitol	+	+	−
D-Sorbitol	+	+	−
Amygdalin	−	+	−
Arbutin	−	+	−
Salicin	+	+	−
Cellobiose	+	+	−
Lactose (bovine origin)	+	+	−
Gentiobiose	+	−	−
D-Fucose	+	W	W
L-Fucose	+	W	+
D-Arabitol	+	+	W
L-Arabitol	+	W	+
Potassium gluconate	−	−	+
Potassium 2-ketogluconate	−	−	+
Potassium 5-ketogluconate	−	−	+
Enzyme activity (API ZYM)			
Cystine arylamidase	−	W	−
Trypsin	W	+	+
β-Galactosidase	W	W	+
DNA G+C content (mol%)	68.51	68.47	71.3

Table 2. Cellular fatty acid profiles of strains MWE 3-5^T, HWE 2-02^T and *N. hankookensis* DS-30^T

Strains: 1, MWE 3-5^T; 2, HWE 2-02^T; 3, *N. hankookensis* DS-30^T. −, <1.0 % of the total or not detected.

Fatty acid	1	2	3
Saturated			
$C_{16:0}$	2.64	4.07	−
$C_{17:0}$	1.27	1.70	−
$C_{18:0}$	1.69	4.50	1.30
Unsaturated			
$C_{17:1}\omega8c$	2.88	−	1.28
$C_{18:1}\omega9c$	7.50	5.92	3.54
Branched			
iso-$C_{14:0}$	1.86	2.90	2.14
iso-$C_{15:0}$	6.85	−	−
iso-$C_{16:0}$	56.20	61.50	66.28
iso-$C_{17:0}$	9.10	−	1.24
anteiso-$C_{17:0}$	1.04	2.00	1.64
iso-$C_{18:0}$	1.73	3.02	4.47
10-Methyl fatty acids			
10-Methyl $C_{17:0}$	−	2.07	5.09
10-Methyl $C_{18:0}$	4.86	6.85	7.40
Summed feature			
9*	1.59	1.08	−

*Summed feature 9 consists of 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$.

Description of *Nocardioides conyzicola* sp. nov.

Nocardioides conyzicola (co.ny.zi.co′la. L. n. *Conyza* a botanical genus; L. suff. *–cola* inhabitant, dweller; N.L. n. *conyzicola* inhabitant of *Conyza*, the plant from which the type strain was isolated).

Cells are Gram-stain-positive, aerobic, non-spore-forming, non-motile and curved or straight rods (0.4–0.5 × 0.6–2.2 μm). Colonies are whitish, circular to slightly irregular with entire margins, convex, smooth, and glistening. Optimal growth occurs at 25–30 °C, and at pH 6.0. Growth occurs in the presence of 0–1.0 % (w/v) NaCl, but not at ⩾2.0 % (w/v). Catalase- and oxidase-positive. Nitrate is reduced to nitrite. Data on carbon assimilation (API 20NE) and enzyme activities (API ZYM) are given in Table 1. The diagnostic diamino acid in the cell-wall peptidoglycan is LL-2,6-diaminopimelic acid. The predominant menaquinone is MK-8(H_4) and the major fatty acids are iso-$C_{16:0}$ and 10-methyl $C_{18:0}$. The polar lipids consist of PIM, PG, unidentified phospholipids and unidentified polar lipids.

The type strain is HWE 2-02^T (=KCTC 29121^T=JCM 18531^T), which was isolated from the surface-sterilized root of *Conyza canadensis*. The genomic DNA G+C content of the type strain is 68.47 mol%.

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