# *Nocardioides caricicola* sp. nov., an endophytic bacterium isolated from a halophyte, *Carex scabrifolia* Steud.

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A Gram-staining-positive, coccoid to rod-shaped bacterium, designated strain YC6903$^T$, was isolated from a halophytic plant (*Carex scabrifolia* Steud.) collected from sand dunes at Namhae Island, Korea, and its taxonomic position was investigated by using a polyphasic approach. Strain YC6903$^T$ grew optimally at 30 °C and at pH 8.0. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain YC6903$^T$ belongs to the genus *Nocardioides* in the family *Nocardioidaceae*. Strain YC6903$^T$ was related most closely to *Nocardioides pyridinolyticus* OS4$^T$ (97.0 % 16S rRNA gene sequence similarity), *Nocardioides dokdonensis* FR1436$^T$ (96.6 %), *Nocardioides aquiterrae* GW-9$^T$ (96.6 %) and *Nocardioides hankookensis* DS-30$^T$ (96.6 %). The cell-wall peptidoglycan contained LL-diaminopimelic acid and MK-8($H_4$) was the major respiratory quinone. The mean (±SD) level of DNA–DNA relatedness between strain YC6903$^T$ and *N. pyridinolyticus* OS4$^T$ was 53.5±5.5 %. The predominant cellular fatty acid of strain YC6903$^T$ was iso-$C_{16:0}$ (28.9 %). The DNA G+C content was 71.7 mol%. Phenotypic, phylogenetic and chemotaxonomic data indicated that strain YC6903$^T$ represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides caricicola* sp. nov. is proposed. The type strain is YC6903$^T$ (=KACC 13778$^T$ =DSM 22177$^T$).

The genus *Nocardioides* was proposed by Prauser (1976) and, at the time of writing, comprises 48 recognized species. Members of the genus have been isolated from various environments such as soils, herbage, groundwater, black sand, beach sand, crude oil, a saline lake and seawater (Yoon *et al.*, 2005, 2006a, b, 2007, 2008; Lee, 2007; Lee *et al.*, 2007, 2008; Tóth *et al.*, 2008). During an investigation of the endophytic bacterial community in the roots of a halophyte, *Carex scabrifolia* Steud., growing on sand dunes on Namhae Island, Korea, we isolated a Gram-staining-positive bacterium (strain YC6903$^T$) that showed high levels of 16S rRNA gene sequence similarity to members of the genus *Nocardioides*.

For the isolation of bacteria from the roots of halophytes, the following process was used (Chung *et al.*, 2008). Root pieces were washed several times in running tap water and surface sterilized by stepwise washing in 70 % ethanol for 5 min, 1.0 % NaOCl for 10 min, 70 % ethanol again for about 10 s and finally with sterile distilled water several times. After confirmation of the surface sterility of root segments incubated at 28 °C for 5–6 days on R2A agar (Difco), 1.0 g of dried plant root was ground in 9.0 ml of autoclaved seawater with a sterile mortar and pestle. Serial dilutions were made using the sterilized seawater and 200 µl of the $10^{-3}$–$10^{-5}$ dilutions were spread on 1/10-strength R2A plates and incubated at 25 °C for 1–2 weeks. The purified strains were maintained on marine agar 2216 (MA; Difco) or half-strength R2A agar (1.6 g R2A broth powder supplemented with 1.5 % agar per litre distilled water).

Cell morphology was observed by using a Nikon light microscope and a scanning electron microscope (JSM-6380LV; JEOL) and the presence of flagella was investigated by using a transmission electron microscope (model H-600; Hitachi) with cells grown for 2 days at 30 °C in half-strength

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YC6903$^T$ is FJ750845.

A supplementary figure and a supplementary table are available with the online version of this paper.

R2A broth. The Gram reaction was determined by using the bioMérieux Gram staining kit according to the manufacturer's instructions. Catalase and oxidase tests were performed by the procedures as outlined by Cappuccino & Sherman (2002). Hydrolysis of casein, aesculin, gelatin, starch, L-tyrosine and urea were investigated according to Brown (2007), and hydrolysis of Tweens 20 and 80 according to Atlas (1993). Enzyme activities and other physiological and biochemical properties were determined by using API ZYM, API 20E, API 20NE and API 50 CH test strips at 30 °C according to the manufacturer's instructions (bioMérieux). Growth at 4, 10, 20, 25, 28, 30, 37, 40, 45 and 50 °C and at pH 5.0–12.0 (at intervals of 0.5 pH units, at 30 °C) was tested on half-strength R2A agar plates after 7 days of incubation. Growth under anaerobic conditions was determined on half-strength R2A agar plates for 7 days at 30 °C in an anaerobic Gaspak jar containing an atmosphere of $CO_2$ (Gas-Pack System; Becton Dickinson). Salt tolerance was tested in half-strength R2A broth supplemented with 0–6 % (w/v) NaCl (at 0.5 % intervals) after 7 days of incubation at 30 °C. Duplicate antibiotic-sensitivity tests were performed by using filter-paper discs containing gentamicin (10 μg), penicillin (10 μg), ampicillin (10 μg), rifampicin (30 μg), tetracycline (30 μg), kanamycin (30 μg), vancomycin (30 μg), streptomycin (50 μg) or chloramphenicol (100 μg). Discs were placed on half-strength R2A agar plates spread with strain YC6903$^T$ and related type strains and the plates were incubated at 30 °C for 3 days.

Cells of strain YC6903$^T$ were Gram-positive, non-motile, coccoid to curved rods (0.4–0.6 × 2.0–5.0 μm) (see Supplementary Fig. S1 in IJSEM Online). The strain grew well on half-strength R2A agar, nutrient agar and MA, but did not grow on MacConkey agar. Growth did not occur under anaerobic conditions. The physiological and biochemical characteristics of strain YC6903$^T$ are summarized in the species description below and a comparison of selected characteristics with those of related type strains is given in Table 1.

Cellular fatty acids of strain YC6903$^T$ and of the type strains of three related species of the genus *Nocardioides* were analysed by using colonies grown on half-strength R2A agar plates for 72 h at 30 °C. Analysis of fatty acid methyl esters was performed according to the instructions

**Table 1.** Differential phenotypic characteristics between strain YC6903$^T$ and the type strains of related species of the genus *Nocardioides*

Strains: 1, YC6903$^T$; 2, *N. pyridinolyticus* KCTC 0074BP$^T$; 3, *N. aquiterrae* KCCM 41647$^T$; 4, *N. hankookensis* KCTC 19246$^T$. +, Positive; −, negative; w+, weakly positive. Data for reference strains are from this study unless indicated otherwise.

| Characteristic | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Cell morphology | Rods, cocci | Rods, cocci | Rods, cocci | Rods |
| Cell size (μm) | 0.4–0.6 × 2.0–5.0 | 0.5–0.6 × 1.2–1.6 | 0.8–1.0 × 1.7–2.0 | 0.4–0.8 × 1.5–10.0 |
| Optimal growth temperature (°C) | 30 | 35 | 30 | 25 |
| Motility | − | + | + | − |
| Hydrolysis of: | | | | |
| L-Tyrosine | − | − | − | + |
| Tween 80 | + | − | − | + |
| Tributylin | − | + | + | + |
| Susceptibility to: | | | | |
| Chloramphenicol (100 μg) | + | − | + | + |
| Streptomycin (50 μg) | + | − | + | + |
| Tetracycline (30 μg) | − | + | − | − |
| Ampicillin (10 μg) | − | − | + | − |
| Rifampicin (30 μg) | + | − | − | + |
| API ZYM tests | | | | |
| Cystine arylamidase | − | + | + | + |
| Trypsin | + | + | − | + |
| β-Galactosidase | + | − | + | + |
| Utilization of: | | | | |
| Potassium nitrate | − | + | − | + |
| Aesculin | + | + | − | + |
| Sodium pyruvate | − | w+ | w+ | − |
| Gelatin | − | − | + | − |
| Isolation source | Halophyte | Oil-shale column | Groundwater | Soil |
| DNA G+C content (mol%) | 71.7 | 73[a*] | 73[b] | 71.3[c] |

*Data from: *a*, Yoon *et al.* (1997); *b*, Yoon *et al.* (2004); *c*, Yoon *et al.* (2008).

of the Microbial Identification System (MIDI; Microbial ID). The isomer of diaminopimelic acid in the peptidoglycan was analysed by using TLC according to the method described by Komagata & Suzuki (1987). Isoprenoid quinones were extracted and analysed by using reversed-phase HPLC according to the method described by Komagata & Suzuki (1987). For measurement of the G+C content of the chromosomal DNA, genomic DNA of strain YC6903$^T$ was extracted and purified as described by Ausubel *et al.* (1995). It was then enzymically degraded into nucleosides and the G+C content was determined as described by Mesbah *et al.* (1989). DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain YC6903$^T$ and its closest relative, *Nocardioides pyridinolyticus* OS4$^T$. DNA–DNA hybridization was performed by using the procedure of Ezaki *et al.* (1989). Five replicate hybridizations were conducted for each sample. The DNA relatedness value quoted is expressed as the mean (±SD) of five replicate values.

The major cellular fatty acids (>5.0 %) of strain YC6903$^T$ were iso-$C_{16:0}$ (28.9 %), $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$ (8.1 %), $C_{18:1}\omega5c$ (7.0 %) and $C_{14:0}$ (6.6 %). This cellular fatty acid profile was generally similar to those of closely related species of the genus *Nocardioides*, but was distinguishable from them in terms of the proportion of some fatty acids (see Supplementary Table S1). Anteiso-$C_{15:1}$ (2.5 %) was detected only in strain YC6903$^T$. The peptidoglycan of strain YC6903$^T$ contained LL-2,6-diaminopimelic acid. The major respiratory quinone was MK-8($H_4$).

Extraction of genomic DNA was performed by using a commercial genomic DNA extraction kit (Core Biosystem). The 16S rRNA gene of strain YC6903$^T$ was amplified by PCR from the purified genomic DNA by using primers 27F and 1492R (Lane, 1991). The PCR product was cloned into the TOPO TA vector (Invitrogen) and sequenced. The 16S rRNA gene sequence was compiled by using SeqMan software (DNASTAR) and the sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). A phylogenetic tree was reconstructed by using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA4 program (Tamura *et al.*, 2007) with bootstrap values based on 1000 replications (Felsenstein, 1985). A phylogenetic tree based on the maximum-likelihood algorithm was also reconstructed by using the PHYLIP program, version 3.6 (Felsenstein, 2002). Pair-wise sequence similarity values between strain YC6903$^T$ and the type strains of related bacteria were computed by using a global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007).

The 1401 nt of the 16S rRNA gene sequence of strain YC6903$^T$ was covered by all sequences in the MEGA4 analysis after alignment. Phylogenetic analyses of 16S rRNA gene sequences showed that strain YC6903$^T$ was related closely to species of the genus *Nocardioides* in the family *Nocardioidaceae* (Fig. 1). In the neighbour-joining phylogenetic tree, strain YC6903$^T$ joined the cluster comprising *N. pyridinolyticus*, *Nocardioides hankookensis* and *Nocardioides aquiterrae* with 58 % bootstrap support (Fig. 1). Strain YC6903$^T$ exhibited 16S rRNA gene sequence

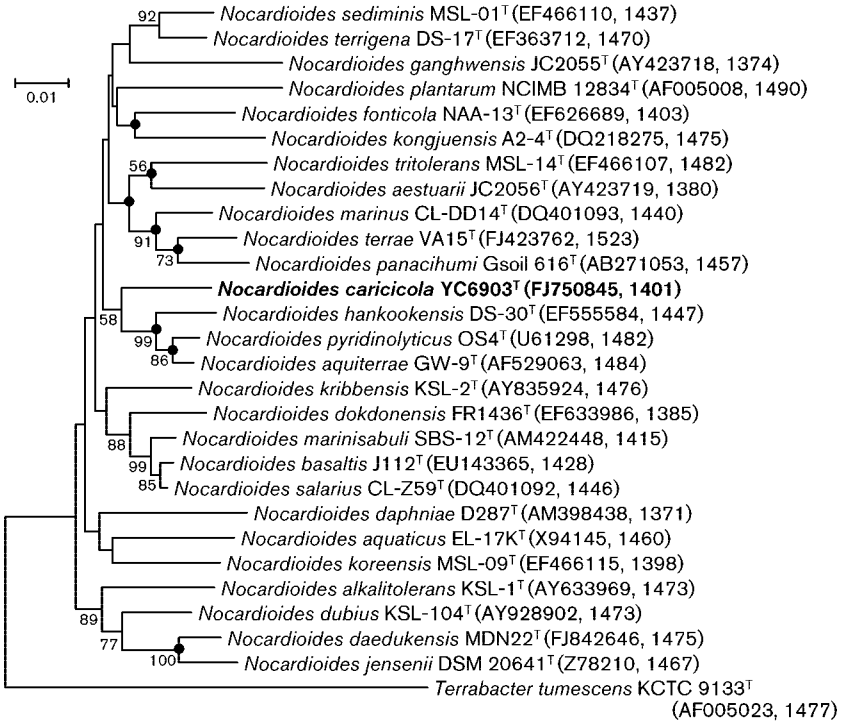


**Fig. 1.** Phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationship between strain YC6903$^T$ and related taxa. The phylogenetic tree was reconstructed by using the neighbour-joining method and Jukes–Cantor evolutionary distance matrix data obtained from aligned nucleotides (number of nucleotides and GenBank accession numbers in parentheses). Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. Bar, 1 substitution per 100 nt positions.

similarity values of 97.0, 96.6, 96.6 and 96.6 % with the type strains of *N. pyridinolyticus*, *Nocardioides dokdonensis*, *N. aquiterrae* and *N. hankookensis*, respectively. It shared less than 96.1 % 16S rRNA gene sequence similarity with other taxa used in the phylogenetic analysis (Fig. 1). The DNA G+C content of strain YC6903$^T$ was 71.7 mol%. The level of DNA–DNA relatedness between strain YC6903$^T$and *N. pyridinolyticus* OS4$^T$, the type strain of the most closely related species of the genus *Nocardioides*, was 53.5±5.5 %.

Results of 16S rRNA gene sequence similarity calculations and phylogenetic analysis (Fig. 1) clearly indicated that strain YC6903$^T$ belongs to the genus *Nocardioides*. Strain YC6903$^T$ is distinguishable from recognized species of the genus *Nocardioides* based on several phenotypic characteristics (Table 1). Antibiotic sensitivity, fatty acid composition (Table S1), phylogenetic analysis and differential phenotypic properties distinguished strain YC6903$^T$ from recognized species of the genus *Nocardioides*. We thus suggest that strain YC6903$^T$ represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides caricicola* sp. nov. is proposed.

### Description of *Nocardioides caricicola* sp. nov.

*Nocardioides caricicola* [ca.ri.ci′co.la. L. n. *carex -icis* reed-grass, rush or sedge, and also a botanical genus name (*Carex*); L. suff. *-cola* (from L. n. *incola*) inhabitant, dweller; N.L. n. *caricicola Carex*-dweller, isolated from *Carex scabrifolia* Steud.].

Cells are Gram-stain-positive, non-spore-forming, coccoid to curved rods (0.4–0.6 × 2.0–5.0 μm). Colonies are smooth, convex, glistening, circular, white and 0.5–0.7 mm in diameter after 5 days of incubation at 30 °C on half-strength R2A agar. Grows at 10–45 °C (optimum, 30 °C) and pH 7.0–9.0 (optimum, pH 8.0). Growth occurs in the absence of NaCl but not in the presence of >0.5 % (w/v) NaCl in half-strength R2A broth. Growth does not occur under anaerobic conditions. Resistant to ampicillin (10 μg) and tetracycline (30 μg), but susceptible to penicillin (10 μg), gentamicin (10 μg), vancomycin (30 μg), kanamycin (30 μg), rifampicin (30 μg), streptomycin (50 μg) and chloramphenicol (100 μg). Oxidase-negative and catalase-positive. Hydrolyses aesculin, casein, Tween 20 and Tween 80, but not gelatin, starch, carboxymethylcellulose, xylan or L-tyrosine. Cannot use the single carbon sources available in the API 20NE test strips. Urea is not hydrolysed. Indole and $H_2S$ are not produced. Tryptophan deaminase is not produced. Nitrate is reduced to nitrogen gas. Acid is produced from D-glucose (API 20E kit). In the API ZYM kit, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, acid phosphatase, β-galactosidase, α-glucosidase and β-glucosidase, but negative for lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The cell-wall peptidoglycan contains LL-2,6-diaminopimelic acid. The major quinone is MK-8($H_4$). The major fatty acids are iso-$C_{16:0}$, $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$, $C_{18:1}\omega5c$ and $C_{14:0}$. The DNA G+C content of the type strain is 71.7 mol%.

The type strain, YC6903$^T$ (=KACC 13778$^T$ =DSM 22177$^T$), was isolated from a halophyte, *Carex scabrifolia* Steud., growing on sand dunes on Namhae Island, Korea.

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