**Nocardioides salsibiostraticola** sp. nov., isolated from biofilm formed in coastal seawater

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A Gram-staining-positive, non-motile, aerobic, non-spore-forming and short rod-shaped bacterial strain, PAMC 26527T, was isolated from biofilm formed in coastal seawater of the Norwegian Sea. Analysis of the 16S rRNA gene sequence of strain PAMC 26527T revealed a clear affiliation with the genus *Nocardioides*. Based on phylogenetic analysis, strain PAMC 26527T showed the closest phylogenetic relationship with *Nocardioides caricicola* YC6903T with 16S rRNA gene sequence similarity of 96.3%. Strain PAMC 26527T grew in the presence of 0–5.0% sea salts. The optimum temperature and pH for growth were 20 °C and pH 7.5. The major cellular fatty acids of strain PAMC 26527T were iso-C16:0, C17:1ω8c and C18:1ω9c and the major isoprenoid quinone was MK-8(H4). Cell-wall analysis showed that strain PAMC 26527T contained LL-diaminopimelic acid. The genomic DNA G+C content was 69.3 mol%. The combined phenotypic, chemotaxonomic and phylogenetic data showed that strain PAMC 26527T could be clearly distinguished from recognized members of the genus *Nocardioides*. Thus, strain PAMC 26527T should be classified as representing a novel species in the genus *Nocardioides*, for which the name *Nocardioides salsibiostraticola* sp. nov. is proposed. The type strain is PAMC 26527T (=KCTC 29158T=JCM 18743T).

The genus *Nocardioides*, first described by Prauser (1976), belongs to the family *Nocardioidaceae*, in the class *Actinobacteria*. At the time of writing, the genus comprised 58 recognized species (List of Prokaryotic Names with Standing in Nomenclature; http://www.bacterio.cict.fr) with the description of a further five species awaiting publication. Most species of the genus *Nocardioides* have been isolated from non-saline environments, including herbage, oil shale column, groundwater, organisms from freshwater and soil (Collins et al., 1994; Yoon et al., 1997, 2004; Tóth et al., 2008; Lee et al., 2012). However, 11 species have been discovered from various marine environments: *Nocardioides marinus* (Choi et al., 2007) and *Nocardioides marinquilinus* (Cho et al., 2013) from seawater, *Nocardioides salarius* (Kim et al., 2008) from seawater enriched with zooplankton, *Nocardioides marinisabuli* (Lee et al., 2007), *Nocardioides furvisabuli* (Lee, 2007), *Nocardioides hwasunensis* (Lee et al., 2008), *Nocardioides dokodonensis* (Park et al., 2008) and *Nocardioides basaltis* (Kim et al., 2009) from beach sand, *Nocardioides caricicola* (Song et al., 2011) from a halophyte growing on a sand dune, and *Nocardioides aestuarii* (Yi & Chun, 2004b) and *Nocardioides ganguhwensis* (Yi & Chun, 2004b) from tidal flat sediment.

In this study, we describe a bacterial strain, PAMC 26527T, isolated from biofilm formed in coastal seawater near Ny-Ålesund, Svalbard, Norway. Based on data from the present polyphasic study, we propose that the bacterium represents a novel species in the genus *Nocardioides*.

Strain PAMC 26527T was isolated from biofilm formed on polycarbonate acryl plates (200 × 300 mm) that were submerged into seawater next to a marina at 1 m depth. Biofilms were scraped off and put in 50 ml conical tubes using a sterile razor blade. The biofilm samples were stored at −80 °C as a 30% glycerol stock until further analyses. An aliquot (100 μl) of the glycerol stock of the biofilm sample was spread onto 0.1 × saline R2A agar [tenfold diluted R2A agar (Difco) supplemented with 4% (v/v) sea salts (Sigma)] and incubated aerobically at 10 °C for...
3 weeks. Among 16 strains isolated from the biofilm sample (13 strains belonging to the class Actinobacteria and three strains identified only as members of the phylum Bacteroidetes), one strain assigned to the genus Nocardioïdes showed >3% 16S rRNA gene sequence divergence from the type strains of recognized species of the genus Nocardioïdes. Each colony was picked up using a sterile loop and streaked onto fresh 0.1% saline R2A agar. A single colony was transferred onto a new agar plate, and this step was repeated more than four times to confirm the purity of the strain. After salinity and temperature optima were determined, strain PAMC 26527T was routinely cultured onto modified R2A (Difco) agar [R2A medium supplemented with 0.5% (w/v) sea salts (Sigma)] and in modified R2A broth at 20 °C. The strain was stored in modified R2A broth supplemented with 30% (v/v) glycerol at −80 °C.

DNA was extracted from a single colony by the boiling method (Englen & Kelley, 2000). The crude extracts were used as DNA template for PCRs, which included primers 27F and 1492R (Lane, 1991) and Taq DNA polymerase (Promega). The PCR product was purified by using the AccuPrep PCR purification kit (Bioneer). Direct sequencing of the purified 16S rRNA gene PCR product was performed using sequencing primers (27F, 518F, 808R and 1492R; Lane, 1991; Anzai et al., 1997) in an Applied Biosystems sequencer (Macrogen). The almost-complete 16S rRNA gene sequence (1461 bp) of strain PAMC 26527T was compared against the GenBank and EzTaxon-e databases using BLASTN (Altschul et al., 1997; Kim et al., 2012). Sequence similarity was calculated using the EzTaxon-e server. The 16S rRNA gene sequences of all type strains in the genus Nocardioïdes were obtained from GenBank and the Ribosomal Database Project II database (Cole et al., 2007). Multiple alignment was performed manually according to the 16S rRNA gene secondary-structure information implemented in the PHYDIT program (Jeon et al., 2005). All phylogenetic trees in this study were reconstructed on the basis of a consensus 1270 bp of 16S rRNA gene sequences. An evolutionary distance matrix for the neighbour-joining data was generated according to the model of Jukes & Cantor (1969). Phylogenetic trees based on the neighbour-joining (Saitou & Nei, 1987) and the maximum-parsimony (Fitch, 1971) methods were reconstructed using MEGA5 (Tamura et al., 2011). A phylogenetic tree based on the maximum-likelihood method was reconstructed using PAUP 4.0 (Felsenstein, 1981; Swoford, 1998). The maximum-likelihood method was implemented by using a hierarchical ratio test in MODELTEST, version 3.60 (Posada & Crandall, 1998). For robustness of tree topology, bootstrap analyses were based on 1000 replications for the neighbour-joining and maximum-parsimony methods and 100 replications for the maximum-likelihood method.

N. caricicola YC6903T (Song et al., 2011) was used as a reference strain in all morphological, physiological and biochemical analyses. Unless otherwise specified, all morphological and physiological characteristics described hereafter were based on cultures grown for 10 days in modified R2A broth at the optimal temperature of each strain, 20 °C for strain PAMC 26527T and 30 °C for N. caricicola YC6903T. All morphological and physiological tests of the reference strain were also carried out at 20 °C to compare with strain PAMC 26527T at the same incubation temperature. Morphological and physiological tests were performed as follows. Gram-staining was performed using the method of Smibert & Krieg (1994). Cell motility was observed by the hanging drop method (Suzuki et al., 2001). Cell morphology was examined by light microscopy and transmission electron microscopy (EX2; JEOL). Cells used for microscopic observation were grown for 4 days at 20 °C in modified R2A broth. Anaerobic growth was tested on modified R2A agar using the GasPak anaerobic system (BBL) at 20 °C for 14 days. Catalase activity was determined by bubble formation on addition of 3% (v/v) H2O2 and oxidase activity was determined using 1% (w/v) tetramethyl-p-phenylenediamine (Cappuccino & Sherman, 2002). The temperature range for growth was determined by assessing changes in OD600 over time in modified R2A broth at different temperatures (5–45 °C, at increments of 5 °C) for 10 days. The pH range for growth was also determined by assessing changes in OD600 in modified R2A broth adjusted to different pH (pH 5.0–10.0, at increments of 0.5 pH units), incubating at 20 °C for 10 days. Buffers were used for pH range experiments (citrate phosphate buffer for pH 5.0–6.0, potassium phosphate buffer for pH 5.0–8.0, Tris/HCl buffer for pH 8.5–9.0 and glycine–NaOH buffer for pH 9.5–10.0, each at a final concentration of 50 mM) (Lee et al., 2012). Salt tolerance test was carried out on the basis of changes in OD600 in R2A broth [pH adjusted to 7.5 using MOPS (Sigma) at a final concentration of 0.1 M] (Scheidle et al., 2011) supplemented with 0–10% NaCl or sea salts (Sigma) at increments of 0.5% (w/v) at 20 °C for 10 days.

Tests for hydrolysis of gelatin, starch, and Tweens 20, 40, 60 and 80 were performed according to Hövik Hansen & Sorheim (1991) after 10 days of incubation on modified R2A agar. Decomposition of casein, hypoxanthine and xanthine were determined according to the protocols described by Smibert & Krieg (1994). Cultures of strain PAMC 26527T and the reference strain used for carbon source utilization tests were grown in modified R2A broth for 10 days at the optimal growth temperature for each strain. Cells were harvested by centrifugation, subsequently washed with modified basal broth medium and resuspended in the same broth medium (Bhadra et al., 2005). Carbon source utilization was tested using a modified basal broth medium without NaCl and CaCl2 containing, per litre distilled water: 11.8 g KCl, 2.26 g MgCl2·6H2O, 2.97 g MgSO4·7H2O, 0.2 g NaNO3, 0.2 g NH4Cl and 0.05 g yeast extract; pH 7.5) supplemented with 0.5% sea salts (Hwang & Cho, 2006). Each carbon source was added at a final concentration of 0.4%. Carbon utilization was determined as negative when growth was equal to, or less than, that in the negative control with no carbon source.
Growth was measured by monitoring changes in OD_{600} (Ultraspec 2000; Pharmacia Biotech) after 14 days' incubation at 20 °C. In addition, other biochemical activities of strain PAMC 26527^T were determined by using API 20NE and API ZYM (bioMérieux) kits according to the manufacturer’s instructions. For the tests using API ZYM and API 20NE kits, strain PAMC 26527^T and the reference strain were cultivated in modified R2A broth at 20 and 30 °C, respectively, for 10 days. Cells were harvested by centrifugation and resuspended in suspension medium (bioMérieux) at a cell density corresponding to tube no. 5 and no. 1 of the MacFarland scale of standard opacities (for API ZYM and API 20NE tests, respectively). Antibiotic-sensitivity tests were performed by using autoclaved filter paper discs containing ampicillin (10 µg), chloramphenicol (100 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin (10 µg), rifampicin (30 µg), streptomycin (50 µg), tetracycline (30 µg) and vancomycin (30 µg). Autoclaved

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<th>Table 1. Selected physiological and biochemical characteristics of strain PAMC 26527^T and the type strains of other phylogenetically related species of the genus Nocardioides</th>
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<td>Strains: 1, PAMC 26527^T (data from this study); 2, N. cariiciola YC6903^T (this study; Song et al., 2011); 3, N. salarius CL-Z59^T (Kim et al., 2008); 4, N. dokdonensis FR1436^T (Park et al., 2008). The results for strain PAMC 26527^T and N. cariiciola YC6903^T shown below were obtained at 20 °C. Salt tolerance of these two strains was 0–5 % (optimum 0.5 %). The two strains were positive for oxidase, catalase activity and hydrolysis of gelatin and casein, but negative for hydrolysis of Tweens 20 and 80, starch, hypoxanthine and xanthine. In API ZYM kits, the two strains were positive for esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase activities, but negative for valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, z-mannosidase and z-fucosidase activities. In API 20 NE kits, the two strains were positive for nitrate reduction and hydrolysis of aesculin. The two strains were positive for utilization of cellobiose, fructose, galactose, glucose, mannitol, raffinose, salicin, trehalose, lactose, l-arabinose, l-rhamnose, N-acetyl-D-glucosamine, pyruvic acid, sucrose and l-proline, but negative for acetate, citrate, inositol, maleic acid and succinate. +, Positive; −, negative; W, weakly positive; NA, no available data. In the total polar lipids profile, phosphatidylglycerol (PG), unidentified phospholipids (PL1 and PL3), and an unidentified lipid (L2) were detected from the first two strains.</td>
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<td>DNA G+C content (mol%)</td>
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*Results at 30 °C.
†Result from Song et al. (2011).
distilled water was also used as a negative control. Bacterial
lawns were made by spreading the strains onto modified
R2A agar plates. Discs containing antibiotics or autoclaved
distilled water were placed on the bacterial lawn and the
plates were incubated at 20 °C for 10 days. Cells were
determined as susceptible when clear zones of complete
growth inhibition were observed in bacterial lawns (Enger
et al., 1987; Song et al., 2011).

In all chemotaxonomic analyses (isoprenoid quinones,
fatty acids, total polar lipids, cell-wall diamino acids),
strain PAMC 26527T and the reference strain were cultured
in modified R2A broth at 20 °C for 10 days (i.e. early
stationary phase growth). Cells were collected by centrifuga-
tion, subsequently washed with 0.2 μm-filtered auto-
claved PBS, and the collected cells were lyophilized for 24 h
for further analyses (Hwang et al., 2006). Isoprenoid
quinones were extracted according to the method of
Minnikin et al. (1984) and were analysed by HPLC
(Collins, 1985). Polar lipids were extracted by the method
described by Minnikin et al. (1984), examined by two-
dimensional TLC and identified by spraying with detection
reagents (Komagata & Suzuki, 1987). The detection
reagents used were 0.2% ninhydrin in 1-butanol for
aminolipids, 10% phosphomolybdic acid in 95% ethanol
for total lipids, molybdenum blue (Sigma) for phospho-
lipids and ω-naphthol-sulfuric acid for glycolipids. All TLC
plates were scanned immediately after the end of reaction
with detection reagents. The fatty acid methyl esters in
whole cells of strain PAMC 26527T and N. cariciola
YC6903T grown in modified R2A broth at 20 °C for
10 days were analysed by GC according to the instruction
of the Microbial Identification System at the Korean
Culture Center of Microorganisms (KCCM) in Seoul,
Korea. Fatty acid methyl esters of N. cariciola YC6903T
grown at 30 °C were also analysed. The cell-wall diamino
acids were extracted and examined by TLC as described by
Staneck & Roberts (1974). For DNA G+C content
analysis, DNA was extracted by the method of Marmur
(1961) and was analysed by HPLC (HP 100; Hewlett
Packard) as described by Mesbah et al. (1989). Lambda
phage DNA was analysed together for calibration.

Cells of strain PAMC 26527T grown for 4 days at 20 °C
in modified R2A broth were short rods, 0.5–0.6 μm in
width and 1.1–2.1 μm in length (Fig. S1 available in IJSEM
Online). Strain PAMC 26527T grown for 7 days on
modified R2A agar at 20 °C formed circular, white, convex
and opaque colonies that were 0.9–1.2 mm in diameter.
Cells were Gram-staining-positive, aerobic, non-motile and
non-spore-forming. The optimal temperature for growth
was 20 °C. No growth occurred at 0 or 30 °C after 10 days
incubation. The pH range for growth was from pH 6.0 to
9.5 and the optimal pH was 7.5. Strain PAMC 26527T
was not able to grow in R2A broth in the presence of NaCl as
a single salt, but grew in the presence of sea salts (Sigma); salt
tolerance ranged from 0 to 5.0%. The results of oxidase
catalase and tests were positive. Gelatin and casein were
hydrolysed, but starch, Tween 20, 40, 60 and 80,
hyoxanthine and xanthine were not. Other morphological
and physiological characteristics of strain PAMC 26527T
are given in the species description below and a
comparison of selected morphological, physiological and
biochemical characteristics of strain PAMC 26527T and N.
cariciola YC6903T is provided in Table 1.

Phylogenetic analyses based on 16S rRNA gene sequences
showed that strain PAMC 26527T belonged to the genus
Nocardioides. Strain PAMC 26527T was related most closely
to N. cariciola YC6903T with a 16S rRNA gene sequence
similarity of 96.3%. 16S rRNA gene sequence similarity
between strain PAMC 26527T and the type strains of other related
species of the genus Nocardioides was ≤95.6%. In all of the
phylogenetic trees, strain PAMC 26527T formed a branch
distinct from N. cariciola YC6903T (Fig. 1). Thus, the low
similarity value (i.e. ≤96.3%) with recognized species of the
genus Nocardioides and the phylogenetic position
of strain PAMC 26527T showed that the strain could be
assigned to a novel species in the genus Nocardioides.
The DNA G+C content of strain PAMC 26527T was
69.3 mol%, within the range (67–75 mol%; Prauser, 1976;
Yoon et al., 2006; Cho et al., 2013) reported for recognized
species of the genus Nocardioides.

The major isoprenoid quinone of strain PAMC 26527T was
MK-8(H4) as seen in the other members of the genus
Nocardioides (Holt et al., 1994; Busse & Schumann 1999).
Cell-wall peptidoglycan analysis indicated that L-L-
diaminopimelic acid was present in strain PAMC 26527T. These
results also support that strain PAMC 26527T is a member of the
genus Nocardioides (Prauser, 1976). The fatty acid
profile was dominated by iso-C_{16:0} (44.5%; Table S1),
which is typically found as a major component in members
of the genus Nocardioides (Yoon et al., 1999). C_{17:1} \text{cis-9c}
(20.0%) and \text{C}_{18:1} \text{cis-9c} (10.4%) were other major
cellular fatty acids of strain PAMC 26527T. The total polar
lipids were composed of phosphatidyglycerol (PG), unidentified
phospholipids (PL1–PL3) and an unidentified lipid (L2)
(Fig. S2).

The following phenotypic characteristics distinguished
strain PAMC 26527T from its closest phylogenetic
neighbour, N. cariciola YC6903T. The optimal
temperature for growth of strain PAMC 26527T was 20 °C
and no growth occurred at 30 °C, which is the optimal
temperature for growth of N. cariciola YC6903T (Song et al.,
2011). Strain PAMC 26527T could be distinguished from
N. cariciola YC6903T by its inability to hydrolyse
Tweens 40 and 60, ability to utilize glycerol, and inability to utilize
mannose, ascorbate and L-L-aspartate as a sole carbon
source. According to API ZYM tests, strain PAMC 26527T
differed from N. cariciola YC6903T by positive results for
lipase (C14) and leucine arylamidase activity and negative
result for \text{x-glucosidase activity (Table 1). In the total fatty}
acid profile, strain PAMC 26527T contained three times
more unsaturated fatty acid C_{17:1} \text{cis-9c} (20.0%) than N.
cariciola YC6903T. In addition, the hydroxyl fatty acid
C_{17:0} \text{3-Oh} (3.4%) was found in strain PAMC 26527T but

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was undetected in *N. caricicola* YC6903<sup>T</sup>. On the other hand, the branched fatty acid iso-C<sub>17:0</sub> was undetected in strain PAMC 26527<sup>T</sup> but present (1.5%) in *N. caricicola* YC6903<sup>T</sup> (Table S1). In the total polar lipid profile, strain PAMC 26527<sup>T</sup> differed from *N. caricicola* YC6903<sup>T</sup> by the absence of unidentified lipid L1 in strain PAMC 26527<sup>T</sup> and the presence of a different unidentified phospholipid (PL2) in strain PAMC 26527<sup>T</sup> (Fig. S2).

In conclusion, based on the phenotypic, chemotaxonomic and phylogenetic data presented, we suggest that strain PAMC 26527<sup>T</sup> represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides salsibiostraticola* sp. nov. is proposed.

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

*Nocardioides salsibiostraticola* [sal.si.bio.stra.ti’co.la. L. adj. salus salted; Gr. n. bios life; L. n. stratum layer; L. suff. -cola (from L. masc. n. incola) inhabitant; N.L. n. salsibiostraticola inhabitant of a salted biofilm].

After 7 days’ incubation on modified R2A agar at 20 °C, colonies are circular, convex, opaque, white and 0.9–1.2 mm in diameter. Growth occurs at 5–25 °C (optimum 20 °C) and at pH 6.0–9.5 (optimum pH 7.5). No growth occurs at 30 °C. Growth occurs in the presence of 0–5.0% (w/v) sea salts (optimum 0.5%). Positive for Gram-staining, and catalase and oxidase activity. Gelatin and casein are hydrolysed, but starch, Tweens 20, 40, 60 and 80, hypoxanthine and xanthine are not. According to API ZYM kits, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, but negative for valine arylamidase, cystine arylamidase, -galactosidase, -glucosidase, -glucuronidase, -glucosidase, -inositol, and -mannosidase and -fucosidase activities. In API 20NE kits, nitrate is reduced to nitrogen gas and aesculin is hydrolysed, but indole production, fermentation of glucose, hydrolysis of gelatin, and enzyme activity of arginine dihydrolase, -galactosidase and urease are absent. In sole carbon utilization tests, cellobiose, fructose, galactose, glucose, glycerol, lactose, mannitol, pyruvic acid, raffinose, salicin, sucrose, trehalose, L-arabinose, L-rhamnose, L-proline and N-acetyl-D-glucosamine are utilized, but acetate, ascorbate, citrate, inositol, maleic acid, mannose, succinate and DL-aspartate are not utilized. Susceptible to all antibiotics used in this study. The major isoprenoid quinone is MK-8(H4). The cell-wall contains LL-diaminopimelic acid. The polar lipids detected are phosphatidylglycerol (PG), unidentified phospholipids (PL1–PL3) and one unidentified lipid (L2).

The type strain is PAMC 26527<sup>T</sup> (=KCTC 29158<sup>T</sup>=JCM 18743<sup>T</sup>), isolated from biofilm formed in coastal seawater of the Norwegian Sea. The genomic DNA G+C content of the type strain is 69.3 mol%.

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### References


