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 maringuillus (Cho et al., 2013) from seawater, Nocardioides salarius (Kim et al., 2008) from seawater enriched with zooplankton, Nocardioides marini-sabuli (Lee et al., 2007), Nocardioides furvisabuli (Lee, 2007), Nocardioides hwasonensis (Lee et al., 2008), Nocardioides dokdonensis (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, 2004a) and Nocardioides ganghwensis (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 20 days. 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 20 days.
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 *Nocardioides* showed >3% 16S rRNA gene sequence divergence from the type strains of recognized species of the genus *Nocardioides*. 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, 800R 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 *Nocardioides* 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 PHYLIP (Fitch, 1971) methods were reconstructed using MEGA5 (Tamura et al., 2011). 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 method (Fitch, 1971) methods were reconstructed using PAUP 4.0 (Felsenstein, 1981; Swofford, 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

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Biofilm formed in coastal seawater</td>
<td>Halophyte growing on sand dunes</td>
<td>Seawater enriched with zooplankton</td>
<td>Sand sediment from a beach</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Short rods</td>
<td>Curved rods</td>
<td>Short rods</td>
<td>Short rods</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>0.5–0.6 × 1.1–2.1</td>
<td>0.4–0.6 × 1.5–4.4</td>
<td>0.6–1.6 × 0.3–0.6</td>
<td>0.6–0.9 × 1.2–1.8</td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>6.0–9.5 (7.5)</td>
<td>5.5–9.5 (7.5)</td>
<td>6–10 (6–7)</td>
<td>5–10 (7)</td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tween 40</td>
<td>–</td>
<td>+</td>
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<td>NA</td>
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<td>Tween 60</td>
<td>–</td>
<td>+</td>
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<td>Susceptibility to:</td>
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<td></td>
<td></td>
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<tr>
<td>Ampicillin (10 μg)</td>
<td>+</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>Tetracycline (30 μg)</td>
<td>+</td>
<td>–</td>
<td>(W*)</td>
<td>NA</td>
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<td>Sole carbon utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mannose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Glycerol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<td>NA</td>
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<td>DL-Aspartate</td>
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<td>+</td>
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<td>NA</td>
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<tr>
<td>API ZYM test results</td>
<td></td>
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<tr>
<td>Acid phosphatase</td>
<td>+</td>
<td>–</td>
<td>(+*)</td>
<td>+</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>–</td>
<td>(W*)</td>
<td>–</td>
</tr>
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<td>Lipase (C14)</td>
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<td>Leucine arylamidase</td>
<td>+</td>
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<td>+</td>
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<td>α-Glucosidase</td>
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<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>Unidentified phospholipids (PL)</td>
<td>PL1, PL2, PL3</td>
<td>PL1, PL3</td>
<td>NA</td>
</tr>
<tr>
<td>Unidentified lipids (L)</td>
<td>L2</td>
<td>L1, L2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>69.3</td>
<td>71.3 (71.7†)</td>
<td>73.3</td>
<td>69.1</td>
</tr>
</tbody>
</table>

*Results at 30 °C.
†Result from Song et al. (2011).
Cells of strain PAMC 26527 T grown for 4 days at 20 °C 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 26527 T 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 centrifugation, subsequently washed with 0.2 μm-filtered autoclaved 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% phosphomolybdcic acid in 95% ethanol for total lipids, molybdenum blue (Sigma) for phospholipids and α-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 26527 T and N. carlicicola YC6903 T 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. carlicicola YC6903 T grown at 30 °C were also analysed. The cell-wall diamino acids were extracted and examined by TLC as described by Stanec & 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 26527 T 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 26527 T 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 26527 T 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 and catalase tests were positive. Gelatin and casein were hydrolysed, but starch, Tween 20, 40, 60 and 80, hypoxanthine and xanthine were not. Other morphological and physiological characteristics of strain PAMC 26527 T are given in the species description below and a comparison of selected morphological, physiological and biochemical characteristics of strain PAMC 26527 T and N. carlicicola YC6903 T is provided in Table 1.

Phylogenetic analyses based on 16S rRNA gene sequences showed that strain PAMC 26527 T belonged to the genus Nocardioides. Strain PAMC 26527 T was related most closely to N. carlicicola YC6903 T with a 16S rRNA gene sequence similarity of 96.3%. 16S rRNA gene sequence similarity between strain PAMC 26527 T and the type strains of other related species of the genus Nocardioides was <95.6%. In all of the phylogenetic trees, strain PAMC 26527 T formed a branch distinct from N. carlicicola YC6903 T (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 26527 T showed that the strain could be assigned to a novel species in the genus Nocardioides.

The DNA G+C content of strain PAMC 26527 T 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 26527 T 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 LL-diaminopimelic acid was present in strain PAMC 26527 T. These results also support that strain PAMC 26527 T is a member of the genus Nocardioides (Prauser, 1976). The fatty acid profile was dominated by iso-C16:0 (44.5%; Table S1), which is typically found as a major component in members of the genus Nocardioides (Yoon et al., 1999), C17:1ω8c (20.0%) and C18:1ω9c (10.4%) were other major cellular fatty acids of strain PAMC 26527 T. The total polar lipids were composed of phosphatidylglycerol (PG), unidentified phospholipids (PL1–PL3) and an unidentified lipid (L2) (Fig. S2).

The following phenotypic characteristics distinguished strain PAMC 26527 T from its closest phylogenetic neighbour, N. carlicicola YC6903 T. The optimal temperature for growth of strain PAMC 26527 T was 20 °C and no growth occurred at 30 °C, which is the optimal temperature for growth of N. carlicicola YC6903 T (Song et al., 2011). Strain PAMC 26527 T could be distinguished from N. carlicicola YC6903 T by its inability to hydrolyse Tween 40 and 60, ability to utilize glycerol, and inability to utilize mannose, ascorbate and L-aspartate as a sole carbon source. According to API ZYM tests, strain PAMC 26527 T differed from N. carlicicola YC6903 T by positive results for lipase (C14) and leucine arylamidase activity and negative result for α-glucosidase activity (Table 1). In the total fatty acid profile, strain PAMC 26527 T contained three times more unsaturated fatty acid C17:1ω8c (20.0%) than N. carlicicola YC6903 T. In addition, the hydroxyl fatty acid C17:0 3-ΟΗ (3.4%) was found in strain PAMC 26527 T but...
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. *salsus* 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, pyrroline and cyanide 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, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities, but negative for valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-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, β-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(H<sub>4</sub>). 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**


