

Rhodococcus aerolatus sp. nov., isolated from subarctic rainwater

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A Gram-stain-positive, rod-shaped and non-motile strain, designated PAMC 27367^T, was isolated from rainwater collected on the Bering Sea. Analysis of the 16S rRNA gene sequence of the strain showed an affiliation with the genus *Rhodococcus*. Phylogenetic analyses revealed that strain PAMC 27367^T formed a robust clade with the type strains of *Rhodococcus rhodnii*, *Rhodococcus aetherivorans* and *Rhodococcus ruber* with 16S rRNA gene sequence similarities of 96.3%, 95.8% and 95.5%, respectively. Cells of the strain grew optimally at 25 °C and at pH 6.5–7.0 in the presence of 0–2% (w/v) sea salts. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and three unknown phospholipids. The major cellular fatty acids (>10%) were iso-C_{16:0}, C_{17:1ω8c} and 10-methyl C_{17:0}. Cell wall analysis showed that strain PAMC 27367^T contained meso-diaminopimelic acid. The genomic DNA G+C content was 77.1 mol%. Based on the phylogenetic, chemotaxonomic and phenotypic data presented here, we propose a novel species with the name *Rhodococcus aerolatus* sp. nov., with PAMC 27367^T (=KCTC 29240^T=JCM 19485^T) as the type strain.

Since the genus *Rhodococcus* was proposed by Zopf (1891), extensive data from polyphasic taxonomic studies have improved classification for members of this genus (Goodfellow *et al.*, 1998; Gürtler & Seviour, 2010; Jones & Goodfellow, 2012; Kämpfer *et al.*, 2014). Currently, the genus *Rhodococcus* is placed in the family *Nocardiaceae* within the order *Actinomycetales* (Jones & Goodfellow, 2012). At the time of writing, the genus comprises 37 species with validly published names (List of Prokaryotic Names with Standing in Nomenclature; <http://www.bacterio.net/rhodococcus.html>). Members of novel species of the genus *Rhodococcus* have been isolated from a broad range of habitats including soil, freshwater, indoor air, marine sediment and herbivorous dung (Jones & Goodfellow, 2012). Some members of the species *Rhodococcus* exhibit diverse metabolic activities, including the degradation of aliphatic and aromatic hydrocarbons (Yoon *et al.*, 2000a, b; Goodfellow *et al.*, 2004) and

the production of storage compounds (Warhurst & Fewson, 1994; Bunch, 1998), while some are known as opportunistic animal and plant pathogens (Scott *et al.*, 1995; Temmerman *et al.*, 2000). In the present study, a bacterial strain isolated from rainwater on the subarctic Bering Sea was subjected to polyphasic taxonomic analysis and subsequently allocated to the genus *Rhodococcus*.

A rainwater sample was collected onboard R/V *Araon* using a sterile precipitate-collector above the Bering Sea (64.57° N 170.20° W) during the Ship-borne Pole-to-Pole Observations (SHIPPO) expedition in July 2012. An aliquot (100 µl) of rainwater was spread on marine agar 2216 (MA; Difco) and incubated at 25 °C under aerobic conditions for one week. Strain PAMC 27367^T was isolated and subsequently streaked onto MA. The purification procedure was repeated four times. The strain was maintained on MA at 25 °C and preserved in marine broth 2216 (MB; Difco) supplemented with 30% (v/v) glycerol at –80 °C.

Three type strains of species of the genus *Rhodococcus* including *Rhodococcus rhodnii* KCCM 41081^T, *Rhodococcus ruber* KCCM 41053^T and *Rhodococcus aetherivorans* DSM 44752^T were obtained from the Korean Culture Center of

Abbreviation: FAME, fatty acid methyl esters.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PAMC 27367^T is KM044053.

Two supplementary figures are available with the online Supplementary Material.

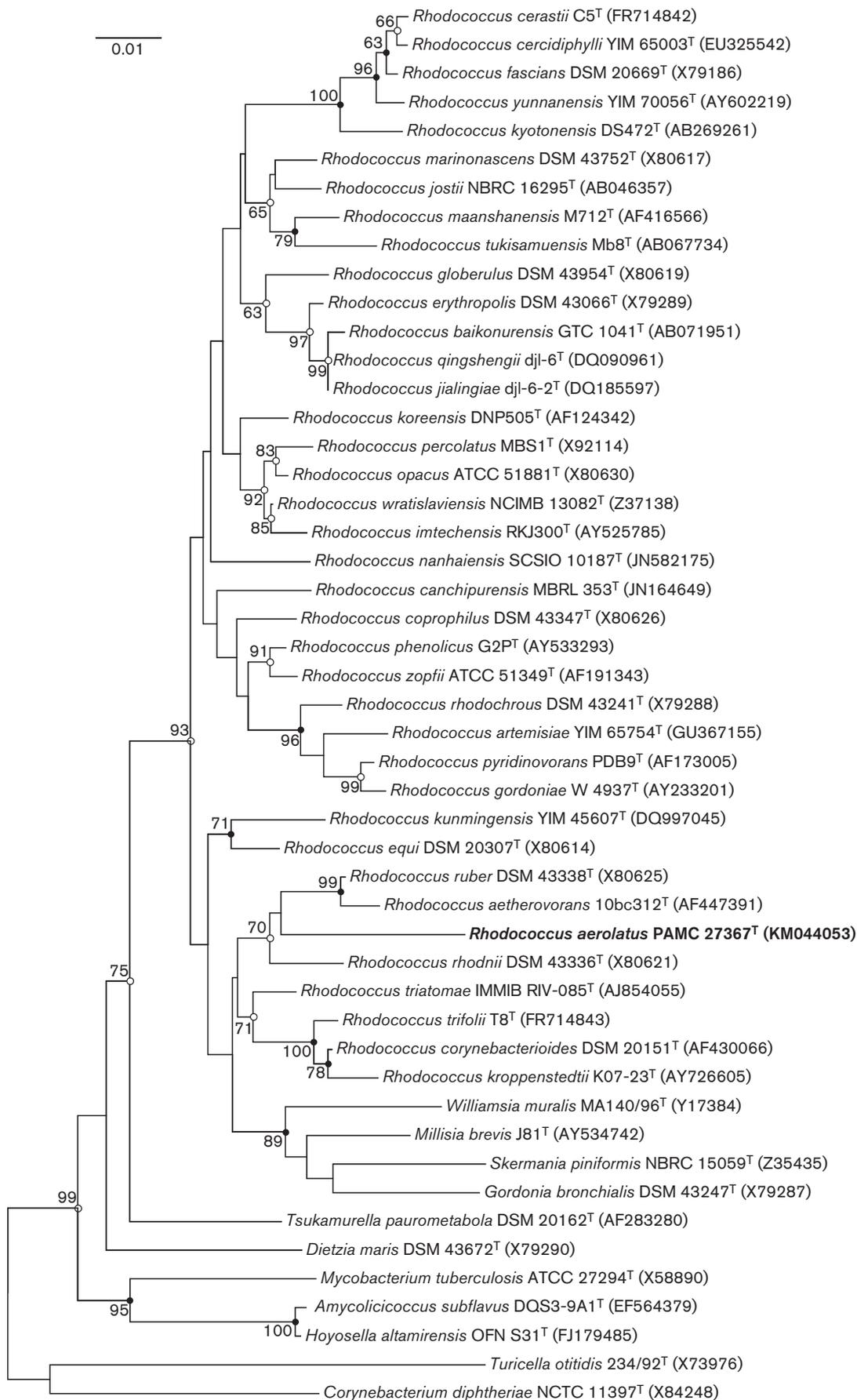


Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain PAMC 27367^T and related species on the basis of 16S rRNA gene sequences. Only bootstrap values above 60% are shown (1000 resamplings) at the branching points. Solid circles indicate that the corresponding nodes were also obtained in both the maximum-likelihood and the minimum-evolution trees, while open circles indicate that they were obtained in the latter tree only. *Turicella otitidis* 234/92^T (X73976) and *Corynebacterium diphtheriae* NCTC 11397^T (X84248) were used as an outgroup. Bar, 0.01 nt substitution per site.

Microorganisms (KCCM) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in order to compare their physiological and chemotaxonomical characteristics with those of strain PAMC 27367^T. Growth of each strain was tested on MA and tryptic soy agar (TSA; Difco) under various temperature and salinity conditions (see below). Unless specified otherwise, all characteristics of strain PAMC 27367^T and *R. rhodnii* KCCM 41081^T were based on cultures grown aerobically on MA at 25 °C for 5–7 days, whereas those of *R. ruber* KCCM 41053^T and *R. aetherivorans* DSM 44752^T were determined on TSA at 30 °C for 5–7 days. Under these conditions, all strains

appeared to be in the mid- to late-exponential phases of growth.

For 16S rRNA gene amplification by PCR, DNAs were extracted from a single colony by the boiling method (Englen & Kelley, 2000). The crude extracts served as DNA templates for PCR, which included *Taq* DNA polymerase (Promega) and primers 27F and 1492R (Lane, 1991). The PCR product was purified with shrimp alkaline phosphatase and exonuclease I (USB), incubated at 37 °C for 30 min and subsequently at 80 °C for 10 min. Direct sequencing of the purified PCR product was performed using sequencing primers (27F, 337F, 518R, 785F and 1492R; Lane, 1991;

Table 1. Differential characteristics of strain PAMC 27367^T and related species of the genus *Rhodococcus*

Strains: 1, *Rhodococcus aerolatus* sp. nov. PAMC 27367^T; 2, *R. rhodnii* KCCM 41081^T; 3, *R. aetherivorans* DSM 44752^T; 4, *R. ruber* KCCM 41053^T. Data were obtained in this study, unless indicated otherwise. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4
Temperature range for growth (°C) (optimum)	10–30 (25)	10–35 (30)	10–40 (35)	10–40 (35)
Salinity range for growth (% w/v) (optimum)	0–7.0 (0–2.0)	0–12.0 (0–2.5)	0–12.0 (0.5–1.5)	0–12.0 (0.5–1.0)
pH range for growth (optimum)	5.0–8.0 (6.5–7.0)	5.0–8.0 (6.5–7.0)	6.0–8.5 (7.0)	5.5–8.5 (6.0–7.0)
API ZYM tests				
Acid phosphatase	–	–	+	+
Esterase (C4)	+	w	–	+
Esterase lipase (C8)	w	w	–	+
Naphthol-AS-BI-phosphohydrolase	–	+	+	+
Valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α- and β-glucosidases	–	–	+	–
API 20NE tests				
Arginine dihydrolase	–	w	w	–
Aesculin hydrolysis	–	w	w	–
Gelatin hydrolysis	+	–	–	–
Nitrate reduction	–	–	+	–
Urease	+	+	–	w
Utilization as sole carbon source				
N-Acetyl-D-glucosamine	–	–	+	–
L-Arabinose	+	–	–	–
Ascorbate	–	–	+	w
Cellobiose	+	–	–*	–
D-Galactose	+	–	+	+*
Glycerol	–	–	+	+
Inositol	–	–	+*	+*
D-Mannitol	–	–*	+	w
DNA G + C content (mol%)	77.1	66†	71.6	65–69†

*Result opposite to that of Jones & Goodfellow (2012).

†Data from Jones & Goodfellow (2012).

Anzai *et al.*, 1997) with an Applied Biosystems sequencer (ABI 3730XL) at Cosmo Genetech (Seoul, Korea). The almost complete 16S rRNA gene sequence (1421 bp) of strain PAMC 27367^T was obtained and analysed using BLAST searches against the GenBank and EzTaxon databases (Altschul *et al.*, 1990; Kim *et al.*, 2012). The 16S rRNA gene sequences of closely related taxa obtained from the GenBank database were aligned using the RDP aligner (Cole *et al.*, 2014) based on secondary structures. Phylogenetic analysis was performed using the program MEGA 6.0 (Tamura *et al.*, 2013). Distance matrices were calculated according to the Jukes and Cantor model (Jukes & Cantor, 1969). Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1992) and maximum-likelihood methods (Felsenstein, 1981) using bootstrap analyses based on 1000 replications.

The fatty acid methyl esters (FAME) in whole cells of strain PAMC 27367^T and the three type strains of species of the genus *Rhodococcus* grown on MA at 25 °C for 5 days were analysed with GC (model 6890; Agilent Technologies) according to the instructions of the Microbial Identification System (MIDI; version 6.2) with the RTSBA6 database at the KCCM. Analysis of FAME for these four type strains was repeated using a different culture medium (i.e. TSBA) and as described above, except with the TSBA6 database. Chemotaxonomic characterizations of cell-wall diamino acids and polar lipids were analysed using cultures grown for 5 days under the following conditions: strain PAMC 27367^T and *R. rhodnii* KCCM 41087^T on MA at 25 °C; *R. aetherivorans* DSM 44752^T and *R. ruber* KCCM 41053^T on TSA at 30 °C. The cell-wall diamino acids were determined as described by Stanek & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC, and identified using the procedures described by Minnikin *et al.* (1984). Genomic DNAs of PAMC 27367^T and *R. aetherivorans* DSM 44752^T were extracted using a commercial kit (DNeasy Blood & Tissue kit; Qiagen). The DNA G + C content was determined by HPLC analysis (Tamaoka & Komagata, 1984), which was carried out by the identification service of the KCCM.

Tests for the phenotypic characteristics of strain PAMC 27367^T were performed in duplicate along with the three type strains of species of the genus *Rhodococcus*, with experiments repeated on different days. Gram staining was performed using the method of Smibert & Krieg (1994). Cell morphology was examined by transmission electron microscopy (EX2; JEOL). The presence of endospores was assessed using malachite green staining (Smibert & Krieg, 1994). Anaerobic growth was tested in an Anaerobic jar containing an AnaeroPak (Mitsubishi Gas Chemical) at 25 °C for 2 weeks. The temperature range for growth was examined on the basis of colony formation on MA and TSA incubated at 5–45 °C in increments of 5 °C. The pH range (pH 5.0–10.0, at intervals of 0.5 pH unit) for growth was determined by assessing changes in OD₆₀₀ in pH-buffered MB (Hwang & Cho, 2008) for strain PAMC 27367^T and *R. rhodnii* KCCM 41087^T or tryptic soy broth

(Difco) for *R. aetherivorans* DSM 44752^T and *R. ruber* KCCM 41053^T using citric acid-phosphate buffer for pH 5.0, MES for pH 5.5–6.5, MOPS for pH 7.0–7.5, AMPD for pH 8.0–9.5 and CAPS for pH 10.0, each at a final concentration of 50 mM at 25 °C for up to 10 days. Salt tolerance was determined by assessing changes in OD₆₀₀ at 25 °C using synthetic ZoBell broth (1 l⁻¹ distilled water: bacto peptone, 5 g; yeast extract, 1 g; ferric citrate, 0.1 g) supplemented with 0–10% (at intervals of 0.5%), 12, 15, 18, 20, 23 and 25% (w/v) of sea salts (Sigma).

Catalase and oxidase tests were performed according to the methods described by Smibert & Krieg (1994) and Cappuccino & Sherman (2002), respectively. Decomposition of casein, hypoxanthine and xanthine was determined as described by Smibert & Krieg (1994). Hydrolysis of gelatin, starch, Tweens 20, 40, 60 and 80 was investigated as described by Hansen & Sørheim (1991). In addition, other enzyme activities were assayed using the API ZYM and API 20NE kits (bioMérieux) according to the manufacturer's instructions, except that the cell suspension was prepared as described by Hwang *et al.* (2009). Carbon utilization was tested according to the protocol of Bruns *et al.* (2001) with a final concentration of 0.4% (w/v) carbon source. Carbon utilization was scored as negative when growth was equal to, or less than, that in the negative control with no carbon source present. Growth was measured by monitoring changes in the OD₆₀₀ for 20 days at 25 °C.

Phylogenetic analyses based on the 16S rRNA gene sequence showed that strain PAMC 27367^T was affiliated with the genus *Rhodococcus* (Figs 1 and S1, available in the online Supplementary Material). Strain PAMC 27367^T was most closely related to *R. rhodnii* (96.3% sequence similarity) and then to *R. aetherivorans* (95.8%) and *R. ruber* (95.5%). The tree topologies inferred from the neighbour-joining and the minimum-evolution methods revealed that strain PAMC 27367^T formed a robust clade with *R. rhodnii*, *R. aetherivorans* and *R. ruber*, while the clade was relatively weak in the maximum-likelihood tree (Figs 1 and S1). The low 16S rRNA gene sequence similarities (<97%; Rosselló-Mora & Amann, 2001) between strain PAMC 27367^T and species of the genus *Rhodococcus* with validly published names, and the phylogenetic position of strain PAMC 27367^T indicated that our strain represents a novel species of the genus *Rhodococcus*.

The morphological, physiological and biochemical characteristics of strain PAMC 27367^T are given in the species description and presented in Table 1. No significant differences were found in the fatty acid profiles of cells grown on MA and TSA for each type strain (Table 2). The major fatty acids (>10%) of strain PAMC 27367^T were iso-C_{16:0} (25.4–27.8%), C_{17:1}ω8c (21.5–22.2%) and 10-methyl C_{17:0} (13.6%; Table 2). The fatty acids C_{16:0}, C_{18:1}ω9c and 10-methyl C_{18:0} are known to be present in major amounts in species of the genus *Rhodococcus* (Jones & Goodfellow, 2012), and these were also found to be present in strain PAMC 27367^T (Table 2). However, the

Table 2. Cellular fatty acid content of strain PAMC 27367^T and other related species of the genus *Rhodococcus*

Strains: 1, *Rhodococcus aerolatus* sp. nov. PAMC 27367^T; 2, *R. rhodnii* KCCM 41081^T; 3, *R. aetherivorans* DSM 44752^T; 4, *R. ruber* KCCM 41053^T. Fatty acids of cells grown on marine agar (MA) or tryptic soy broth agar (TSBA) at 25 °C for 5 days were analysed. Values are percentages of total fatty acids. tr, Trace amounts (<1%), –, not detected.

Fatty acid	MA				TSBA			
	1	2	3	4	1	2	3	4
Saturated								
C _{14:0}	tr	2.3	1.7	3.0	2.3	1.7	1.8	2.4
C _{15:0}		–	–	–	8.8	7.2	2.6	1.6
C _{16:0}	2.6	26.4	23.0	22.0	5.9	21.4	23.5	28.5
C _{17:0}	6.4	4.2	3.2	2.5	7.1	5.8	3.0	1.9
C _{18:0}	2.3	tr	1.2	1.8	1.7	1.2	2.2	3.4
C _{19:0}	–	–	–	–	–	–	–	1.0
C _{20:0}	–	–	–	–	–	–	2.0	–
Unsaturated								
C _{17:1} ω8c	21.5	1.9	3.6	4.1	22.2	8.3	–	1.4
C _{17:1} ω6c	3.5	–	–	tr	–	–	–	–
C _{18:1} ω9c	4.0	2.2	9.8	13.6	3.7	7.8	15.6	22.7
Branched								
iso-C _{14:0}	–	–	–	–	2.0	–	–	–
iso-C _{16:0}	27.8	tr	–	–	25.4	–	–	–
iso-C _{16:1} H*	1.7	–	–	–	–	–	–	–
iso-C _{18:0}	1.3	–	–	tr	–	–	–	–
Hydroxy								
C _{17:0} 3-OH	1.9	–	–	–	–	–	–	–
Methyl								
10-Methyl C _{17:0}	13.6	17.1	2.1	4.2	13.6	9.7	1.7	tr
10-Methyl C _{18:0} (TBSA†)	2.3	22.9	17.5	18.9	2.9	22.6	27.6	17.7
Summed features‡								
3 (C _{16:1} ω6c and/or C _{16:1} ω7c)	tr	15.2	31.6	22.1	2.1	12.8	20.1	16.2
6 (C _{19:1} ω9c and/or C _{19:1} ω11c)	–	1.9	1.1	1.4	–	1.5	–	–
9 (10-methyl C _{16:0} and/or iso-C _{17:1} ω9c)	tr	1.9	1.7	2.2	1.5	–	–	1.1

*The position of the double bond, indicated by a capital letter, is unknown.

†Tuberculostearic acid.

‡Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system.

amounts of C_{16:0} (2.6–5.9%) and 10-methyl C_{18:0} (2.3–2.9%) in strain PAMC 27367^T were substantially lower than those in closely related species of the genus *Rhodococcus* (17.5–28.5%; Table 2). In addition, the amount of iso-C_{16:0} obviously differentiated strain PAMC 27367^T (25.4–27.8%) from other species of the genus *Rhodococcus* (<1%; Table 2). Strain PAMC 27367^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid, and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and three unknown phospholipids as the major polar lipids (Fig. S2), supporting the attribution of strain PAMC 27367^T to the genus *Rhodococcus* (Jones & Goodfellow, 2012). The genomic DNA G+C content of strain PAMC 27367^T was 77.1 mol%, which was higher than those of phylogenetically related species of the genus *Rhodococcus* (65–71.6 mol%;

Table 1) or those of species of the genus *Rhodococcus* with validly published names (63–73 mol%; Jones & Goodfellow, 2012).

Strain PAMC 27367^T can be distinguished from phylogenetically related species of the genus *Rhodococcus* by its abilities to hydrolyse gelatin, to utilize L-arabinose as the sole carbon source, and its inability to produce naphthol-AS-BI-phosphohydrolase (Table 1). In addition, combinations of some phenotypic characteristics can serve to differentiate all the species in the *Rhodococcus* clade that encompassed strain PAMC 27367^T (Table 1, Fig. 1).

The phylogenetic, chemotaxonomic and phenotypic data obtained in this study indicate that strain PAMC 27367^T should be assigned to a novel species in the genus *Rhodococcus*, for which the name *Rhodococcus aerolatus* sp. nov. is proposed.

Description of *Rhodococcus aerolatus* sp. nov.

Rhodococcus aerolatus (ae.ro.la'tus. Gr. n. *aer* air; L. part. adj. *latus* carried; N.L. part. adj. *aerolatus* airborne).

Gram-staining-positive, strictly aerobic and non-spore-forming rods approximately 0.6–0.9 µm wide and 1.7–8.2 µm long. After 7 days on MA at 30 °C colonies are ivory, circular and convex, and approximately 0.5 mm in diameter. Grows at 10–30 °C (optimum, 25 °C) and at pH 5.0–8.0 (optimum, pH 6.5–7.0). Growth occurs at 0–7.0 % (w/v) sea-salt concentration (optimum, 0–2.0 % w/v). Negative for oxidase. Positive for catalase. Gelatin, and Tweens 20, 40, 60 and 80 are hydrolysed, but starch, casein, hypoxanthine and xanthine are not. In API ZYM tests: esterase (C4) is positive; esterase lipase (C8) and leucine arylamidase are weakly positive; *N*-acetyl-β-glucosaminidase, acid and alkaline phosphatases, α-chymotrypsin, cystine arylamidase, α-fucosidase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase are negative. In API 20NE tests, gelatin hydrolysis, indole production and urease are positive, whereas arginine dihydrolase, glucose fermentation, aesculin hydrolysis and nitrate reduction are negative. L-Arabinose, cellobiose, D-galactose, glucose, mannose, L-proline, pyruvate, succinate, trehalose and sucrose are utilized as the sole carbon source, but acetate, *N*-acetyl-D-glucosamine, ascorbate, fructose, glycerol, lactose, D-mannitol, raffinose, L-rhamnose and salicin are not. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and three unknown phospholipids. Major fatty acids are iso-C_{16:0}, C_{17:1}ω8c and 10-methyl C_{17:0}. The cell wall contains *meso*-diaminopimelic acid.

The type strain, PAMC 27367^T (=KCTC 29240^T=JCM 19485^T), was isolated from rainwater collected from above the Bering Sea. The genomic DNA G+C content of the type strain is 77.1 mol%.

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