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ORIGINAL PAPER

Lacinutrix jangbogonensis sp. nov., a psychrophilic bacterium isolated from Antarctic marine sediment and emended description of the genus *Lacinutrix*

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Abstract A Gram-negative, strictly aerobic, non-motile, rod-shaped and psychrophilic bacterial strain, PAMC 27137^T, was isolated from the marine sediment of the Ross Sea, Antarctica. Strain PAMC 27137^T was observed to grow at 4–10 °C, at pH 6.5–7.5 and in the presence of 2.5–4.0 % (w/v) sea salts. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain PAMC 27137^T belongs to the genus *Lacinutrix* showing the high similarities with *Lacinutrix mariniflava* JCM 13824^T (97.6 %) and *Lacinutrix algicola* JCM 13825^T (97.1 %). Genomic relatedness analyses based on the average nucleotide identity and the genome-to-genome distance showed that strain PAMC 27137^T is clearly distinguished from the most closely related *Lacinutrix* species. The major fatty acids (>5 %) were identified as iso-$C_{15:1}$ G (19.9 %), iso-$C_{15:0}$ (19.3 %), iso-$C_{17:0}$ 3-OH (11.3 %), summed feature 9 ($C_{16:0}$ 10-methyl and/or iso-$C_{17:1}$ ω9*c* as defined by MIDI, 9.1 %), iso-$C_{15:0}$ 3-OH (7.5 %), and anteiso-$C_{15:1}$ A (5.8 %). The polar lipids were found to consist of phosphatidylethanolamine, an unidentified aminolipid, an unidentified amino-phospholipid, and five unidentified phospholipids. The major respiratory quinone was identified as MK-6. The genomic DNA G+C content was determined to be 32.1 mol%. Based on the data from this polyphasic taxonomic study, strain PAMC 27137^T is considered to represent a novel species of the genus *Lacinutrix*, for which the name *Lacinutrix jangbogonensis* sp. nov. is proposed. The type strain is PAMC 27137^T (=KCTC 32573^T=JCM 19883^T).

Keywords *Lacinutrix jangbogonensis* sp. nov. · Polyphasic taxonomy

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Introduction

The genus *Lacinutrix* in the family *Flavobacteriaceae* was established with *Lacinutrix copepodicola* as the type species (Bowman and Nichols 2005). At the time of writing, the genus *Lacinutrix* contains four validly named species (http://www.bacterio.net/lacinutrix.html) and the type strains of this genus were all isolated from polar environments. *L. copepodicola,* was isolated from the surface of the copepod species

Paralabidocera antarctica dwelling in Ace Lake in the Vestfold Hills, an ice-free region of eastern Antarctica (Bowman and Nichols 2005). The type strains of *Lacinutrix algicola* and *Lacinutrix mariniflava* were isolated from a marine red alga collected at Marian Cove on King George Island in the Antarctic (Nedashkovskaya et al. 2008) and *Lacinutrix himadriensis* from a marine sediment in Kongsfjorden, Svalbard in the Arctic Ocean (Srinivas et al. 2013). Members of the genus *Lacinutrix* were one of the major groups of cultivable protease-producing bacteria obtained from sediments of Maxwell Bay, KingGeorge Island, Antarctica suggesting an ecological role in degrading sedimentary organic nitrogen (Zhou et al. 2013). In this study, a psychrophilic bacterium was isolated from Antarctic marine sediment and subjected to polyphasic taxonomic analysis. The strain, PAMC 27137^T, is considered to represent a novel species of the genus *Lacinutrix*, for which the name *Lacinutrix jangbogonensis* sp. nov. is proposed here.

Materials and methods

Isolation and maintenance of the strain

Sediment samples were collected at a depth of 156 m from Ross Sea in the Southern Ocean (74°38′46.20″S, 164°13′24.60″E) on February 06, 2011 by using a box corer. The sediment samples were suspended in 20 % glycerol and preserved at −80 °C until use. For cultivation, serially diluted aliquots (100 μL) of the sample suspensions were spread on marine R2A (mR2A) agar plates [R2A (Difco) 18.2 g and sea salts (Sigma) 40 g per 1 L distilled water] and incubated at 10 °C for 15 days. A total of 15 colonies with different morphologies were picked and streaked on mR2A plates three or more times to obtain pure cultures. All strains were deposited at the polar and alpine microbial collection (PAMC; Lee et al. 2012) of Korea Polar Research Institute. Strains were preserved as glycerol suspensions (20 % in distilled water, v/v) at −80 °C. In the present study, one of these strains, PAMC 27137^T, was routinely cultured on MA at 10 °C. *L. mariniflava* JCM 13824^T and *L. algicola* JCM 13825^T were obtained from the Japan Collection of Microorganisms (JCM) and used as reference strains following cultivation under comparable conditions as strain PAMC 27137^T.

Molecular analysis

For 16S rRNA gene sequencing, genomic DNA was extracted by using a Mini Tissue DNA kit (Cosmogenetech Inc., Korea), amplified with universal primers, 27F and 1492R, and directly sequenced using primers, 27F, 785F, 926R and 1492R (Lane 1991). The almost-complete 16S rRNA gene sequence (1442 nt) of strain PAMC 27137^T was compared with those of all type strains in the EzTaxon database (Chun et al. 2007) and aligned with those of type strains showing high similarities using the PHYDIT (ver. 3.2) program (http://plaza.snu.ac.kr/~jchun/phydit/). Phylogenetic trees were reconstructed using three tree-making algorithms: the maximum-likelihood method using the PhyML (ver 3.0) program (Guindon and Gascuel 2003), the neighbour-joining method (Saitou and Nei 1987) using the PHYLIP package, version 3.5 (Felsenstein 1993), and maximum parsimony (Fitch 1971) using PAUP (ver 4.0) program (Swofford 2002). The tree topologies were evaluated by bootstrap analysis based on 1,000 resamplings.

For investigation of genome relatedness, whole genome sequencing was attempted for strain PAMC 27137^T and the most related *Lacinutrix* species, *L. mariniflava* JCM 13824^T and *L. algicola* JCM 13825^T. Genomic DNAs were extracted using a DNeasy Tissue and Blood Kit (Qiagen) and genome sequencing was performed using the 454 Genome Sequencer FLX+system (Roche) at Macrogen (Seoul, Korea). The degree of pairwise genome-based relatedness was estimated by both an average nucleotide identity (ANI) value, following the BLAST-based ANI calculation method described by Goris et al. (2007), and the genome-to-genome distance calculation (GGDC) method described by Auch et al. (2010). The DNA G+C content for strain PAMC 27137^T was determined by high-performance liquid chromatography (HPLC) analysis (Tamaoka and Komagata 1984) by the identification service of the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea) and directly calculated from its genome sequence.

Cultural, morphological, physiological, and biochemical characteristics

To determine the optimal growth conditions of the three strains investigated in this study, strains were cultured on marine agar (MA, Difco) and mR2A plates

at different temperatures (4, 10, 15, 20, 25, 30, 37, and 42 °C) for 14 days. All three strains grew better at 10 °C on MA plates than mR2A plates. Thus, all characteristics of the strains were acquired (except for sea salt tolerance) from the cultures grown on MA at 10 °C unless indicated otherwise. The response to pH was determined at pH 5–10 (at intervals of 0.5 pH unit) with incubation for 12 days. The following buffers were used to control the pH range: from 5 to 6.5, MES; from 7 to 7.5, MOPS; from 8 to 9.5, AMPD; pH 10, CAPS; each 0.05 M final concentration, with 1× marine broth used as the basic medium. Tolerance to sea salts was determined on ZoBell agar plates (Bacto peptone 5 g, yeast extract 1 g, ferric citrate 0.1 g, Bacto agar 15 g per 1 L distilled water) supplemented with sea salts (Sigma) at 0–25 % (w/v). Anaerobic growth was tested on both MA and MA supplemented with glucose (1 %) and phenol red (0.001 %) in a jar containing an AnaeroPak (Mitsubishi Gas Chemical) for up to 14 days at 10 °C.

Gram staining was carried out using Gram stain kit (Sigma) according to the manufacturer's instruction. Morphology of cells was examined by using transmission electron microscopy (TEM) (JEM1010, JEOL). For TEM, cells were negatively stained with 2.0 % uranyl acetate on a carbon-coated copper grid. Gliding motility was observed using light microscopy (BX51, OLYMPUS) in fresh wet mounts using the hanging drop method (Bernardet et al. 2002).

Biochemical tests and enzyme activities were determined using API ZYM and API 20NE kits (bioMérieux) according to the manufacturer's instructions except that bacterial strains were suspended in distilled water supplemented with 2.5 % (w/v) sea salts. Catalase activity was tested by observing bubble production with 3 % H_2O_2 according to the standard method. Hydrolysis of Tweens 20, 40, 60 and 80 (each of 1 %) and starch (1 %) was tested using MA supplemented with each component according to Smibert and Krieg (1994). Decomposition of hypoxanthine (1 %) and xanthine (1 %) was tested using MA supplemented with each component according to Gordon et al. (1974). DNase activity was determined with DNase test agar (Becton–Dickinson) supplemented with 2.5 % sea salts.

Chemotaxonomy

For cellular fatty acid analysis, strain PAMC 27137^T and reference strains were grown on MA plates at 10 °C for 7 days. Cellular fatty acids were obtained as described by Sasser (1990) following the protocol of Microbial Identification System (MIDI, Inc.). Fatty acid methyl esters were separated by gas chromatography and identified and quantified with the MIDI System software (version 6.2) by the service of the KCCM (Seoul, Korea). Quinones were extracted as described by Collins and Jones (1981) and analysed by HPLC by the service of the KCCM. Polar lipids were extracted, examined by two-dimensional thin layer chromatography (TLC), and identified using the procedures of Minnikin et al. (1984).

Results and discussion

Among 15 strains isolated from Antarctic marine sediment and identified by 16S rRNA gene sequencing, only one strain (designated as PAMC 27137^T) was affiliated with the genus *Lacinutrix*, while the others were affiliated with 7 other genera, including *Bizionia*, *Formosa*, *Loktanella*, *Polaribacter*, *Psychroserpens*, *Shewanella*, and *Winogradskyella* (data not shown). At the 16S rRNA gene sequence level, strain PAMC 27137^T (GenBank accession number KF977035) was found to be closely related to *L. mariniflava*, *L. algicola*, *L. himadriensis*, and *L. copepodicola* with sequence similarity to the respective type strains of 97.6, 97.1, 95.7, and 95.3 %. In addition, PAMC 27137^T formed a monophyletic clade with the type strains of *L. mariniflava* and *L. algicola,* which was consistent in all three tree making analysis (Fig. 1). Thus, strain PAMC 27137^T was characterized simultaneously with *L. mariniflava* JCM 13824^T and *L. algicola* JCM 13825^T for comparisons.

The details of draft genomes for the three strains, which were performed for investigation of genome relatedness, are summarized in Table 1. The ANI values calculated for the estimation of the degree of pairwise genome-based relatedness between strain PAMC 27137^T and the type strains of *L. mariniflava* and *L. algicola* were 85.2 and 85.0 %, respectively (Table S1) and this level is well below the ANI cut-off values (95–96 %) proposed for delineating bacterial species (Goris et al. 2007; Richter and Rosselló-Móra 2009). Consistently, DNA–DNA hybridization values estimated by GGDC were 25.5–26.1 % between strain PAMC 27137^T and the

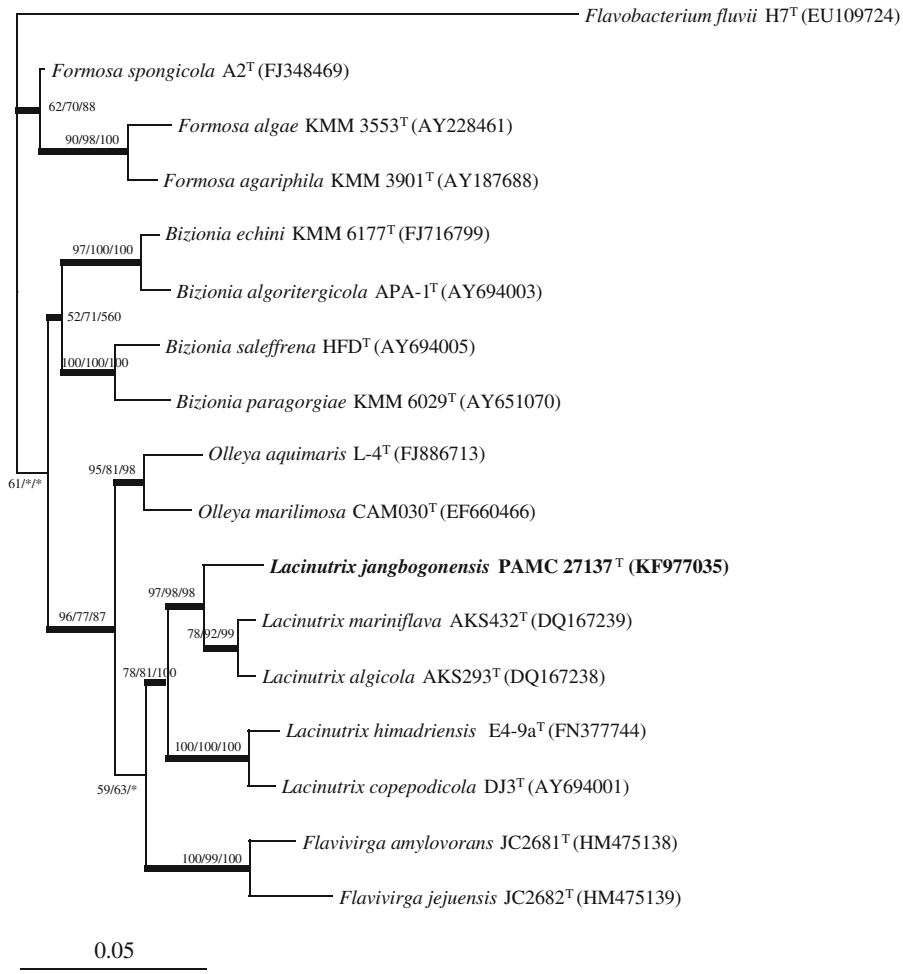

Fig. 1 Phylogenetic tree based on 16S rRNA gene sequences showing the relationships of *Lacinutrix jangbogonensis* sp. nov. PAMC 27137^T with species of the genus *Lacinutrix* and other closely related members of the family *Flavobacteriaceae*. The tree was reconstructed by the heuristic search with the maximum likelihood criterion. Branches that were conserved in maximum likelihood, maximum parsimony, and neighbour-joining analyses are represented by *thick lines*. Percent bootstrap supports (>50 %) are given at each node (ML/MP/NJ). *Flavobacterium fluvii* H7 was used as an outgroup

Table 1 Summary of draft genomes of strain PAMC 27137^T, *Lacinutrix mariniflava* JCM 13824^T, and *Lacinutrix algicola* JCM 13825^T

	Strain 27137^T (PRJNA239392)[a]	L. *mariniflava* JCM 13824^T (PRJNA239393)[a]	L. *algicola* JCM 13825^T (PRJNA239394)[a]
Sequencing platform	454 GS FLX+	454 GS FLX+	454 GS FLX+
Library used	Shotgun	Shotgun	Shotgun
Sequencing coverage	×17	×31	×19
Assembler	Newbler version 2.9	Newbler version 2.9	Newbler version 2.9
Estimated genome size (Mbp)	4.5	4.1	4.0
# of reads	100,890	172,979	100,968
Average read length (bp)	751	743	744
# of large contigs (>500 bp)	100	68	90

[a] Draft genomes for each strain are available under the accession number from PRJNA239392 to PRJNA239394 at the BioProject in NCBI

other type strains (Table S1), indicating that strain PAMC 27137^T is a distinctive *Lacinutrix* species (Rosselló-Mora and Amann 2001). The genomic DNA G+C content of strain PAMC 27137^T was determined to be 33.5 mol% (by a HPLC method) and 32.1 % from the draft genome sequence.

Table 2 Major characteristics that differentiate strain PAMC 27137^T from the type strains of closely related species of the genus *Lacinutrix*

Characteristics	1	2	3
Growth temperature range (°C)[a]	4–10	4–20	4–25
Sea salt tolerance (%)	2.5–4.0	1.0–8.0	1.0–8.0
pH range (optimal pH)[b]	6.5–7.5 (7.0)	6.0–8.0 (7.0)	6.0–8.0 (6.5)
Hydrolysis or decomposition of			
Aesculin	−	+	−
Hypoxanthine	NG	+	+
Skim milk	NG	+	+
Starch	NG	+	+
Tween 40	+	+	+[c]
Tween 60	NG	+	+
Tween 80	+	+[c]	+[c]
Xanthine	−	+	+
Assimilation of			
D-Mannitol	−	+	+
D-Maltose	−	+	−
DNA G+C content (mol%)[d]	32.1	31.8	31.4

Strains: 1, PAMC 27137^T; 2, *L. mariniflava* JCM 13824^T; 3, *L. algicola* JCM 13825^T. Data are from the present study, except where indicated. All strains were non-motile and yellow-pigmented. All strains were positive for the following characteristics: hydrolysis of Tween 20 and gelatin; and presence of catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine-, valine- and cystine arylamidases, trypsin, α-chymotrypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase activities. All strains were negative for the following characteristics: reduction of nitrate to nitrite; indole production; assimilation of D-glucose, L-arabinose, D-mannose, *N*-acetylglucosamine, potassium gluconate, caprate, adipate, malate, citrate, and phenyl acetate in the 20NE system; and presence of arginine dihydrolase, DNase, urease, lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, *N*-acetyl-β-glucosaminidase, and α-fucosidase activities. *NG* no growth (probably due to the substrate added)

[a] Growth temperature range was 0–22 °C for *L. mariniflava* JCM 13824^T and 0–25 °C for *L. algicola* JCM 13825^T according to the results from Nedashkovskaya et al. (2008)

[b] pH range (optimal pH) was 6.0–8.5 (6.5) for *L. mariniflava* JCM 13824^T and 5.5–8.5 (6.5) for *L. algicola* JCM 13825^T according to the results from Nedashkovskaya et al. (2008)

[c] Negative according to the results from Nedashkovskaya et al. (2008)

[d] The genomic DNA G+C content was calculated from its genome sequence

Cultural, physiological, and morphological investigation data for strain PAMC 27137^T revealed that strain PAMC 27137^T grows at 4–10 °C with optimum growth at 10 °C. Strain PAMC 27137^T was found to grow at pH 6.5–7.5, with optimum growth at pH 7.0. Salt was found to be required for growth and the strain tolerated up to 4 % (w/v) sea salts. Strain PAMC 27137^T was found to be strictly aerobic, Gram-negative, and non-motile rods (0.2–0.6 μm wide and 1.5–2.9 μm long) (Supplementary Fig. S1). Colonies were observed to be circular, 1.5–4.0 mm in diameter, shiny, golden-yellow, convex with entire margins after 14 days incubation on MA plates. Physiological, morphological, and biochemical characteristics are summarised in Table 2 and in the species description. A number of phenotypic characteristics clearly distinguish strain PAMC 27137^T from closely related type strains. For example, the growth temperature of PAMC 27137^T ranges from 4 to 10 °C while *L. mariniflava* JCM 13824^T and *L. algicola* JCM 13825^T can grow at up to 20 and 25 °C, respectively. The sea salt tolerance and pH range for growth of PAMC 27137^T is narrow compared to the other two type strains. Hydrolysis or decomposition of hypoxanthine, skim milk, starch, Tween 60, and xanthine by PAMC 27137^T was determined to be negative or the growth was inhibited on the substrate supplemented plates, while the other two type strains were positive for these characteristics.

The predominant fatty acids (>5 %) of strain PAMC 27137^T were identified as iso-$C_{15:1}$ G (19.9 %), iso-$C_{15:0}$ (19.3 %), iso-$C_{17:0}$ 3-OH (11.3 %), summed feature 9 (as defined by MIDI, 9.1 %), iso-$C_{15:0}$ 3-OH (7.5 %) and anteiso-$C_{15:1}$ A (5.8 %) (Table 3). The overall fatty acid composition was similar to that of *L. mariniflava* and *L. algicola*. MK-6 was identified as the only menaquinone present in strain PAMC 27137^T. The polar lipids of strain PAMC 27137^T were found to consist of phosphatidylethanolamine, an unidentified aminolipid, an unidentified aminophospholipid, and five unidentified lipids (Supplementary Fig. S2). This composition was similar to that of *L. mariniflava* and *L. algicola* with respect to the presence of phosphatidylethanolamine, an unidentified aminolipid, and unidentified lipids (Supplementary Fig. S2). However, strain PAMC 27137^T differed from *L. mariniflava* and *L. algicola* in the presence of an unidentified aminophospholipid. In addition, *L. mariniflava* contained one other unidentified lipid and unidentified aminolipid,

Table 3 Fatty acid profiles of strain PAMC 27137^T and other type strains of the genus *Lacinutrix*

Fatty acid	1	2	3
Saturated			
$C_{14:0}$	tr	1.4	tr
$C_{16:0}$	1.4	2.9	1.3
$C_{18:0}$	2.1	1.5	1.3
Unsaturated			
$C_{16:1}\omega5c$	tr	tr	1.2
Iso-$C_{14:0}$	1.4	1.4	1.5
Iso-$C_{15:0}$	**19.3**	**11.3**	**23.0**
Iso-$C_{15:1}$ G	**19.9**	**12.7**	**24.6**
Iso-$C_{16:1}$ H	nd	4.0	3.2
Iso-$C_{16:0}$	2.3	nd	nd
Iso-$C_{16:1}$ G	2.7	nd	nd
Anteiso-$C_{15:0}$	4.2	**9.2**	1.7
Anteiso-$C_{15:1}$ A	**5.8**	**8.1**	2.7
Anteiso-$C_{17:1}$ A	1.0	nd	nd
Anteiso-$C_{17:1}\omega9c$	nd	1.7	tr
Hydroxy			
$C_{15:0}$ 2-OH	1.6	1.8	tr
Iso-$C_{15:0}$ 3-OH	**7.5**	**11.5**	**13.3**
Iso-$C_{16:0}$ 3-OH	4.7	**11.8**	**11.0**
Iso-$C_{17:0}$ 3-OH	**11.3**	**5.3**	4.9
Summed feature 3	1.4	nd	4.0
Summed feature 5	1.5	nd	tr
Summed feature 9	**9.1**	nd	tr

Strains: 1, PAMC 27137^T; 2, *L. mariniflava* JCM 13824^T; 3, *L. algicola* JCM 13825 ^{T}. All strains were grown on MA plates at 10 °C for 7 days. Results are presented as percentages of the total fatty acids. Fatty acids amounting to 5 % or more are highlighted in bold

nd not detected/not reported, *tr* traces (<1 %)

[a] Summed features represent fatty acids that could not be separated by GLC with the MIDI system; summed feature 3 comprises $C_{16:1}$ ω7*c* and/or $C_{16:1}$ ω6*c*, summed feature 5 comprises anteiso-$C_{18:0}$ and/or $C_{18:2}$ ω6, 9*c*, and summed feature 9 comprises $C_{16:0}$ 10-methyl and/or iso-$C_{17:1}$ ω9*c*

while one unidentified lipid was not detected in *L. algicola* (Supplementary Fig. S2).

Overall, the polyphasic taxonomic data obtained in the present study indicate that strain PAMC 27137^T should be assigned to a novel species in the genus *Lacinutrix*, for which the name *L. jangbogonensis* sp. nov. is proposed.

Description of *Lacinutrix jangbogonensis* sp. nov.

Lacinutrix jangbogonensis (N.L. fem. adj. jang.bo.go.nen'sis, of or belonging to Jangbogo, the Korean research station in Antarctica, which is located in coastal margin of Ross Sea where the marine sediment for the isolation of *L. jangbogonensis* was collected).

Cells are Gram-negative, non-motile and rod or curved-rod shaped (0.2–0.6 μm wide and 1.5–2.9 μm long). Colonies on MA plates after 14 days incubation at 10 °C are circular, 1.5–4.0 mm in diameter, shiny, golden-yellow and convex with entire margins. Growth occurs at 4–10 °C (optimum, 10 °C), at pH 6.5–7.5 (optimum, pH 7.0) and in the presence of 2.5–4.0 % (w/v) sea salts. Positive for oxidase and catalase activities. Nitrate is not reduced. Indole is not produced. In the API 20NE system, only gelatin is hydrolyzed. Does not assimilate any of the substrates tested in the API 20NE system. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine-, valine- and cystine arylamidases, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities are present. Tweens 20, 40, and 80 are hydrolyzed. The polar lipids consist of phosphatidylethanolamine, an unidentified aminolipid, an unidentified aminophospholipid, and five unidentified lipids. The menaquinone present is MK-6. The major fatty acids (>5 %) are iso-$C_{15:1}$ G, iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, summed feature 9, iso-$C_{15:0}$ 3-OH, and anteiso-$C_{15:1}$ A. The complete fatty acid composition is given in Table 3. The genomic DNA G+C content of the type strain is 32.1 mol%.

The type strain PAMC 27137^T (=KCTC 32573^T=JCM 19883^T) was isolated from a sediment sample collected from the Ross Sea, Antarctica. The GenBank accession number for the 16S rRNA gene sequence of strain PAMC 27137^T is KF977035.

Emended description of the genus *Lacinutrix*

The description of the genus is as given previously (Bowman and Nichols 2005; Nedashkovskaya et al. 2008; Yi et al. 2012; Srinivas et al. 2013), with the following amendments. Cells are approximately 0.2–0.8 μm wide and 0.7–2.9 μm long.

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