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TAXONOMIC DESCRIPTION
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Pontibacterium granulatum gen. nov., sp. nov., isolated from a tidal flat

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Abstract

A Gram-stain-negative, strictly aerobic, moderately halophilic bacterium, designated A-1^T, was isolated from a tidal flat of the Taean coast in South Korea. Cells were motile rods with a single flagellum showing oxidase-negative and catalase-positive activities and contained poly-β-hydroxyalkanoic acid granules. Growth of strain A-1^T was observed at 20–40 °C (optimum, 30 °C), pH 6.0–10.5 (optimum, pH 7.0) and in the presence of 1.0–6.0 % (w/v) NaCl (optimum, 2.0 %). Strain A-1^T contained $C_{16:0}$, summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) as the major fatty acids. The major polar lipids of strain A-1^T were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The isoprenoid quinones detected were ubiquinone-7 and ubiquinone-8. The G+C content of the genomic DNA was 51.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain A-1^T formed a distinct phylogenetic lineage from other genera within the family *Oceanospirillaceae*. Strain A-1^T shared low 16S rRNA gene sequence similarities with other taxa ($\leq$94.9 %). On the basis of phenotypic, chemotaxonomic and molecular properties, it is clear that strain A-1^T represents a novel genus and species of the family *Oceanospirillaceae*, for which the name *Pontibacterium granulatum* gen. nov., sp. nov. is proposed. The type strain is A-1^T (=KACC 18119^T=JCM 30136^T).

Since the genus *Oceanospirillum* was first described by Hylemon *et al.* [1], many new genera belonging to the family *Oceanospirillaceae* of the phylum *Proteobacteria* have been described. Most genera of the family *Oceanospirillaceae*, except for *Balneatrix alpica*, which was isolated from a spa therapy centre, have been isolated from marine environments [2]. Most members are Gram-staining-negative, strictly aerobic, halotolerant or moderately halophilic, and motile by means of polar flagella, except for members of the genera *Corallomonas* and *Neptunomonas*, which show an anaerobic growth [2–7]. Sea tidal flats, known as getbol in Korea, are broad, low-gradient, coastal, muddy marshes containing many bioresources, and lots of micro-organisms have been isolated from tidal flats in Korea [8–11]. In this study, a member of one more putative new genus belonging to the family *Oceanospirillaceae* was isolated from a tidal flat of the Taean coast and its taxonomic properties were characterized using a polyphasic approach.

Strain A-1^T was isolated from a tidal flat of the Taean coast (36° 48′ 51.5″ N 126° 11′ 08.8″ E), South Korea. A tidal flat sample was serially diluted with artificial seawater (per litre: 20 g NaCl, 2.9 g $MgSO_4$, 4.53 g $MgCl_2.6H_2O$, 0.64 g KCl and 1.75 g $CaCl_2.2H_2O$), spread on marine agar (MA; BD) and incubated at 25 °C for 3 days under aerobic conditions. The 16S rRNA genes from colonies grown on MA were PCR-amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) and double-digested with *Hae*III and *Hha*I, and representative PCR amplicons showing distinct fragment patterns were partially sequenced using the primer 340F (5′-CCTACGGGAGG-CAGCAG-3′). The resulting 16S rRNA gene sequences were compared with those of type strains of validly published species using the Nucleotide Similarity Search program in the EzTaxon-e server (http://www.ezbiocloud.net; [12]). From the 16S rRNA gene sequences analysis, a putative novel strain belonging to the family *Oceanospirillaceae*, designated strain A-1^T, was selected for further phenotypic and phylogenetic analysis. Strain A-1^T was routinely cultured aerobically on MA at 30 °C for 3 days and was stored at −80 °C in marine broth (MB; BD) containing 15 % (v/v) glycerol for long-term preservation. *Corallomonas stylophorae* LMG 25553^T, *Neptuniibacter halophilus* LMG

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Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PHA, poly-b-hydroxyalkanoic acid; RDP, Ribosomal Database Project.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A-1^T is KY658457.
Four supplementary figures and one supplementary table are available with the online Supplementary Material.

25378^T, *Neptuniibacter caesariensis* CECT 7075^T, *Marinobacterium georgiense* KCTC 12422^T and *Oceanospirillum linum* IFO 15448^T were used as reference strains for the comparisons of phenotypic properties and fatty acid compositions.

The 16S rRNA gene of strain A-1^T was further sequenced using the universal primers 518R (5′-ATTACCGCGGCTG CTGG-3′) and 805F (5′-GATTAGATACCCTGGTAGTC-3′). The resulting 16S rRNA gene sequences of strain A-1^T (1472 nucleotides) and closely related type strains were aligned using the fast secondary-structure-aware Infernal aligner available in the Ribosomal Database Project (RDP) [13]. Phylogenetic trees based on the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) algorithms were reconstructed using the MEGA 7 program package [14], and their tree topologies were evaluated through bootstrap analyses based on 1000 replications [15]. The Kimura two-parameter model [16] with the gamma distributed (G) and pairwise deletion options, the Tree-Bisection-Reconnection (TBR) method with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates) and partial deletion options and two-parameter calculation model [16] with gamma distribution with invariant sites (G+I), and the nearest-neighbour-interchange (NNI) heuristic search method and partial deletion options were used for the NJ, MP and ML tree reconstructions, respectively. In addition, the taxonomic assignment of strain A-1^T was performed using the RDP naïve Bayesian rRNA Classifier tool based on an 80 % confidence threshold (http://rdp.cme.msu.edu/classifier; [17]).

A phylogenetic tree based on the ML algorithm showed that strain A-1^T formed a phyletic lineage with the type strains of *Neptuniibacter halophilus* and *Neptuniibacter caesariensis* within the family *Oceanospirillaceae* with a low bootstrap value (23 %) (Fig. 1).

In addition, phylogenetic trees based on the NJ and MP algorithms displayed that strain A-1^T formed phyletic lineages with members of different genera (Figs S1 and S2, available in the online Supplementary Material), and their bootstrap values were also low (30 and 3 %, respectively). The taxonomic analysis using the RDP Classifier tool also indicated that strain A-1^T should be classified as an unclassified *Oceanospirillaceae*, suggesting that strain A-1^T cannot be affiliated with current genus members with validly published names at the species level and could represent a new genus of the family *Oceanospirillaceae*. Comparative analysis based on the 16S rRNA gene sequences revealed that strain A-1^T shared fairly low 16S rRNA gene sequence similarities with members of other all taxa ($\leq$94.9 %), which was below the 95.0 % threshold sometimes referred to for genus delineation [18]. In conclusion, the phylogenetic analyses and 16S rRNA gene sequence similarities suggest that strain A-1^T represents a novel genus of the family *Oceanospirillaceae*.

Growth of strain A-1^T was tested on R2A agar (BD), laboratory-prepared Luria–Bertani (LB) agar, nutrient agar (NA; BD), tryptic soy agar (TSA; BD) and MA (BD) at 30 °C for 3 days. R2A agar, LB agar, NA and TSA were prepared to contain 2 % (w/v) NaCl. Cell morphology was investigated using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) with cells from an exponentially growing culture in MA at 30 °C. The following properties of strain A-1^T and the five reference strains were investigated in parallel under the same conditions. Growth of strain A-1^T at different temperatures (4, 10, 15, 20, 25, 30, 35, 37, 40 and 45 °C) and pH values (pH 5.0–11.0 at 0.5 pH unit intervals) was evaluated in marine broth for 3 days. Marine broth media with pH 5.0–7.0, pH 7.5–9.0 and pH 9.5–11.0 were prepared using Na_2HPO_4/NaH_2PO_4, Tris/HCl and $Na_2CO_3/NaHCO_3$ buffers, respectively [19]. After sterilization (121 °C for 15 min), the pH values were adjusted again if necessary. Growth of strain A-1^T at different NaCl concentrations (0–12 % at 0.5 % intervals, w/v) was tested in marine broth prepared in the laboratory according to the BD formula. The following physiological and biochemical tests were conducted using cells grown on MA for 3 days at 30 °C. Gram staining was investigated using the bioMérieux Gram stain kit according to the manufacturer's instructions. Oxidase activity was evaluated by the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck), and catalase activity was tested by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution [20]. Accumulation of poly-β-hydroxyalkanoic acids (PHAs) was observed using fluorescence microscopy after staining the cells with Nile blue A, as described by Ostle and Holt [21]. Anaerobic growth was assessed on MA with or without 1 % (w/v) glucose or fructose under anaerobic conditions (with 4–10 % CO_2) using the GasPak Plus system (BBL) at 30 °C for 21 days. Hydrolysis of casein, starch, aesculin, tyrosine, urea, gelatin, and Tweens 20 and 80 was tested on MA according to the methods described by Smibert and Krieg [20] by Lányi [22]. Nitrate reduction was assessed according to the method described by Lányi [22]. Additional enzymic activities, biochemical features and oxidation of carbon sources were evaluated using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (Biolog), respectively, according to the manufacturers' instructions, except that cells resuspended in artificial seawater were used as inocula and the test strains were incubated at 30 °C.

Strain A-1^T grew well on MA, but did not grow on LB agar, R2A agar, TSA or NA. Cells of strain A-1^T were rods (0.4–0.6 μm in width and 1.1–1.3 μm in length) motile by means of a single polar flagellum and contained PHA granules inside cells (Fig. S3). Anaerobic growth was not observed after 21 days of incubation at 30 °C. Although some phenotypic properties including tyrosine hydrolysis, nitrate reduction to nitrite and enzyme activity of catalase, alkaline phosphatase and esterase (C4) were in common with reference strains of the family *Oceanospirillaceae*, many other phenotypic properties such as anaerobic growth, flagellum

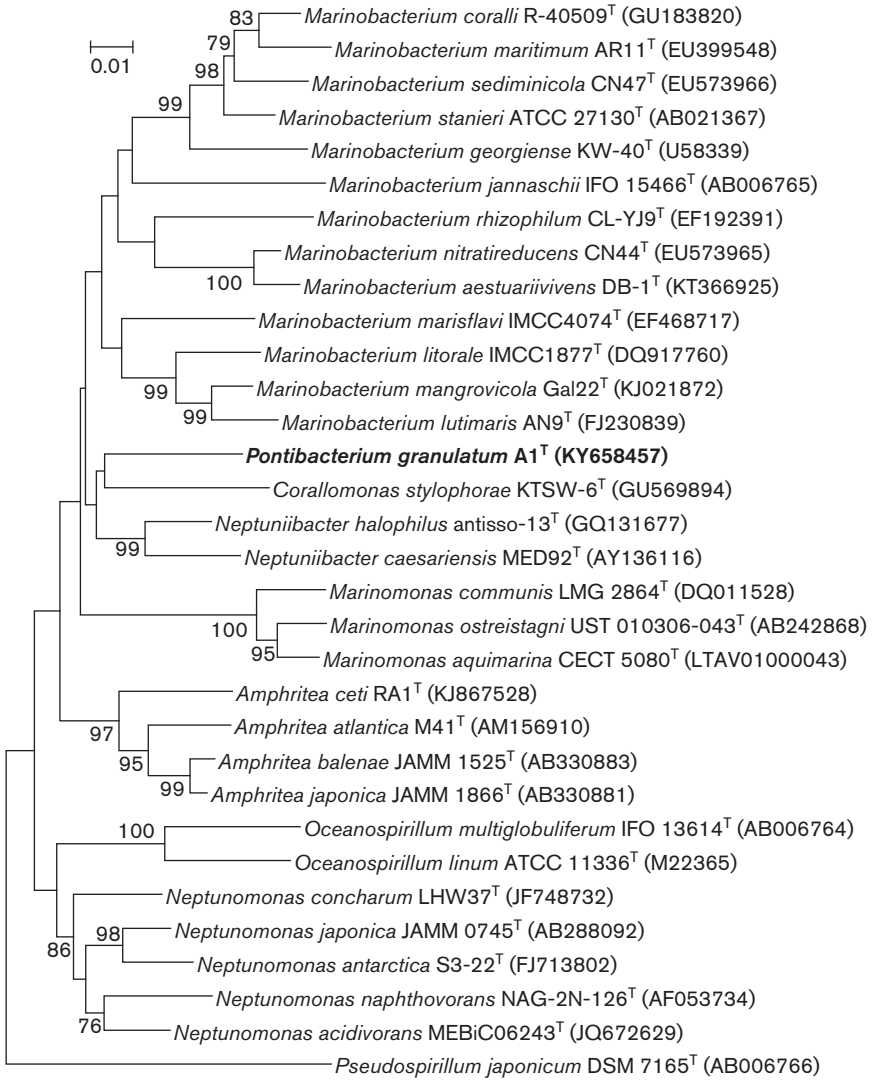

Fig. 1. Maximum-likelihood tree on based on 16S rRNA gene sequences showing the phylogenetic relationships of strain A-1^T and related taxa. Bootstrap values over 70 % are shown at nodes as percentages of 1000 replicates. *Pseudospirillum japonicum* DSM 7165^T (AB006766) was used as an outgroup. Bar, 0.01 changes per nucleotide position.

motility, PHA accumulation, oxidase activity, fermentation of glucose and hydrolysis of aesculin, Tween 20 and gelatin differentiated strain A-1^T from other closely related taxa of the family *Oceanospirillaceae*. Physiological characteristics of strain A-1^T are additionally described in the genus and species descriptions and compared with those of the closely related reference strains in Tables 1 and S1.

Strain A-1^T and the five reference strains were cultivated in marine broth at 30 °C, and microbial cells were harvested at their exponential growth phases for the cellular fatty acid analysis. The cellular fatty acids of microbial cells were saponified, methylated and extracted using the standard MIDI protocol. The fatty acid methyl esters were analysed by a gas chromatography (model 6890; Hewlett Packard) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B; [23]). The polar lipids of strain A-1^T were analysed by TLC using cells harvested during the exponential growth phase according to the procedure described by Minnikin *et al.* [24]. The following reagents were used to detect different polar lipids: 10 % ethanolic molybdophosphoric acid (for total polar lipids), ninhydrin (for aminolipids), Dittmer–Lester reagent (for phospholipids), α-naphthol (for glycolipids) and Dragendorff reagent (for choline). Isoprenoid quinones were extracted according to the method of Minnikin *et al.* [25] and were analysed using a model LC-20A HPLC system (Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250×4.6 mm, Kromasil; Akzo Nobel) as described by

Table 1. Comparisons of phenotypic characteristics of strain A-1^T and related taxa of the family *Oceanospirillaceae*

Strains: 1, A-1^T; 2, *C. stylophorae* LMG 25553^T [5]; 3, *Neptuniibacter halophilus* LMG 25378^T [29]; 4, *Neptuniibacter caesariensis* CECT 7075^T [30]; 5, *M. georgiense* KCTC 12422^T [32]; 6, *O. linum* IFO 15448^T. All data were obtained from this study except the DNA G+C contents. All strains are positive for the following characteristics: nitrate reduction to nitrite, tyrosine hydrolysis and enzyme activity of catalase, alkaline phosphatase and esterase (C4). All strains are negative for the following characteristics: reduction of nitrite to nitrogen gas, indole production, hydrolysis of starch, casein, Tween 80 and urea, enzyme activity of arginine dihydrolase, trypsin, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase and α-fucosidase, and assimilation of L-arabinose and capric acid. +, Positive; −, negative.

Characteristic	1	2	3	4	5	6
Anaerobic growth	−	+	−	−	−	−
Range for growth:						
Temperature (°C)	20–40	15–37	20–37	15–37	4–40	15–37
pH	6.0–10.5	6.5–9.0	6.5–8.5	7.0–9.0	5.5–9.0	6.0–9.0
NaCl (%, w/v)	1.0–6.0	0.5–7.0	0.5–6.0	2.0–6.0	1.0–8.0	2.0–10.0
Motility	+	−	+	+	+	+
PHA granules	+	−	+	+	−	+
Oxidase activity	−	+	+	+	+	+
Fermentation of glucose	−	−	−	−	+	+
Hydrolysis of:						
Aesculin	+	+	−	−	−	−
Tween 20	−	−	−	+	−	+
Gelatin	−	+	+	+	−	−
Enzyme activity (API ZYM) of:						
Esterase lipase (C8)	−	+	+	+	+	+
Lipase (C14)	−	+	−	+	−	+
Leucine arylamidase	+	+	+	+	−	+
Valine arylamidase	+	+	−	+	−	+
Cystine arylamidase,	−	−	−	−	+	+
α-Chymotrypsin	−	−	−	−	−	+
Acid phosphatase and naphthol-AS-BI-phosphohydrolase	−	−	+	+	+	+
α-Galactosidase and α-mannosidase	−	−	−	−	+	−
β-Galactosidase	−	+	−	−	−	−
β-Glucosidase	+	+	−	−	−	−
Assimilation (API 20NE) of:						
D-Glucose	+	−	−	−	+	+
D-Mannose	−	+	+	+	−	−
D-Mannitol, potassium gluconate and malic acid	−	+	+	+	+	+
N-Acetylglucosamine	−	+	−	−	+	+
Maltose	−	−	+	+	−	+
Adipic acid	−	−	−	−	−	+
Trisodium citrate	−	−	−	−	+	+
Phenylacetic acid	−	−	−	−	+	−
DNA G+C content (mol%)	51.5	48.6	54.2	46.6	54.9	49.1*

*Datum from genome sequence of *O. linum* ATCC 11336^T (NZ_MTSD00000000).

Komagata and Suzuki [26]. The DNA G+C content of strain A-1^T was determined by the fluorometric method [27] using SYBR Green I and a real-time PCR thermocycler (Bio-Rad).

The major cellular fatty acids (>10 % of the total fatty acids) of strain A-1^T were $C_{16:0}$, summed feature 3 (comprising $C_{16:1}\omega7c/C_{16:1}\omega6c$) and summed feature 8 (comprising $C_{18:1}\omega7c/C_{18:1}\omega6c$). The overall fatty acid profile of strain A-1^T was similar to those of the reference strains of the family *Oceanospirillaceae*; there were some differences in the respective proportions of some fatty acid components (Table 2). The major polar lipids of strain A-1^T were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Two unknown aminolipids, two unknown phospholipids and two unknown lipids were also detected as minor polar lipids (Fig. S4). The profile of the polar lipids of strain A-1^T was similar to those of members of the genera *Corallomonas* and *Neptuniibacter*, but there were some differences in minor components such as unknown

Table 2. Cellular fatty acid contents (percentages) of strain A-1^T and related taxa of the family *Oceanospirillaceae*

Strains: 1, A-1^T; 2, *C. stylophorae* LMG 25553^T; 3, *Neptuniibacter halophilus* LMG 25378^T; 4, *Neptuniibacter caesariensis* CECT 7075^T; 5, *M. georgiense* KCTC 12422^T; 6, *O. linum* IFO 15448^T. All data were from this study. Data are expressed as percentages of the total fatty acids. Major components (>10.0 %) are highlighted in bold type; –, not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{10:0}$	0.3	–	0.1	0.2	5.2	0.9
$C_{11:0}$	–	–	–	–	–	1.3
$C_{12:0}$	0.3	0.5	0.2	0.1	3.5	7.8
$C_{14:0}$	1.4	1.5	0.5	0.4		1.0
$C_{16:0}$	**18.4**	**16.7**	**17.9**	**18.8**	**24.5**	**20.5**
$C_{17:0}$	–	–	0.3	0.2	–	–
$C_{18:0}$	0.4	1.3	1.5	1.9	–	0.7
10-Methyl $C_{19:0}$	1.2	–	0.7	0.9	–	1.5
Unsaturated						
$C_{15:1}\omega 8c$	0.1	–	–	–	–	–
$C_{16:1}\omega 5c$	0.2	0.1	0.2	–	–	–
$C_{18:1}\omega 9c$	–	0.4	–	–	–	–
11-Methyl $C_{18:1}\omega 7c$	–	0.5	–	–	–	–
$C_{20:1}\omega 7c$	0.1	0.1	0.9	1.2	–	–
Hydroxy						
$C_{10:0}$ 3-OH	9.1	5.1	6.7	7.2	4.3	6.2
$C_{12:1}$ 3-OH	0.1	–	–	–	–	–
$C_{16:0}$ 3-OH	0.2	–	0.2	0.1	–	–
$C_{18:0}$ 3-OH	–	–	0.3	0.2	–	–
Summed features*					–	–
3	**35.3**	**34.5**	**29.7**	**27.5**	**22.4**	**28.3**
4	–	–	–	0.1	–	–
5	–	0.2	0.1	–	–	–
8	**32.9**	**39.1**	**40.7**	**41.2**	**40.1**	**31.8**

*Summed features represent groups of two or three fatty acids that cannot be separated by gas-liquid chromatography with the MIDI system. Summed feature 3, $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$; summed feature 4, iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B; summed feature 5, $C_{18:2}\omega 6,9c$ and/or anteiso-$C_{18:0}$; summed feature 8, $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$.

aminophospholipids, aminolipids and phospholipids [5, 28–30]. The isoprenoid quinones of strain A-1^T detected were ubiquinone-8 (83.3 %) and ubiquinone-7 (16.7 %), which was similar to those of *C. stylophorae* but different from those of other reference strains including *Neptuniibacter halophilus* and *Neptuniibacter caesariensis* containing only ubiquinone-8 [5, 28–31]. The genomic DNA G+C content of strain A-1^T was approximately 51.5 mol%, which was in the range of DNA G+C contents of the reference strains. In conclusion, the physiological and chemotaxonomic features and the phylogenetic inference of strain A-1^T support that strain A-1^T represents a novel species of a new genus of the family *Oceanospirillaceae*, for which the name *Pontibacterium granulatum* gen. nov., sp. nov. is proposed.

DESCRIPTION OF THE GENUS *PONTIBACTERIUM* GEN. NOV.

Pontibacterium (Pon.ti.bac.te′ri.um. L. n. *pontus* the sea; L. neut. n. *bacterium* a small rod; N.L. neut. n. *Pontibacterium* a rod-shaped bacterium isolated from a marine environment).

Cells are Gram-stain-negative, strictly aerobic and motile rods. Catalase activity is positive, while oxidase activity is negative. Cells contain PHA granules. Nitrate is reduced to nitrite, but produce nitrogen gas is not produced. The major fatty acids are $C_{16:0}$, summed feature 3 (comprising $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$) and summed feature 8 (comprising $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$). The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. Major isoprenoid quinones are ubiquinone-7 and ubiquinone-8. Phylogenetically, the genus is a member of the family *Oceanospirillaceae* of the phylum *Proteobacteria*. The type species is *Pontibacterium granulatum*.

DESCRIPTION OF *PONTIBACTERIUM GRANULATUM* SP. NOV.

Pontibacterium granulatum (gra.nu.la′tum. N.L. neut. adj. *granulatum* granulated, containing granules inside cells).

In addition to the characteristics given in the genus description above, this species has the following properties. Colonies on MA are creamy white and irregular circular. Growth occurs at 20–40 °C (optimum, 30 °C), pH 6.0–10.5 (optimum, pH 7.0) and in the presence of 1.0–6.0 % (w/v) NaCl (optimum, 2.0 %). Indole production and fermentation of glucose are not detected. Hydrolysis activities of tyrosine and aesculin occur, but not of starch, casein, Tween 20, Tween 80, gelatin or urea. Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase and β-glucosidase activities are positive, but arginine dihydrolase, esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are negative. Assimilation of D-glucose is positive, but L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid are not assimilated. The major cellular fatty acids (>10 %) are $C_{16:0}$, summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and summed feature 8 (comprising $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$).

The type strain is A-1^T (=KACC 18119^T=JCM 30136^T), isolated from a tidal flat in South Korea. The DNA G+C content is 51.5 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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