Pontibaca methylaminivorans gen. nov., sp. nov., a member of the family *Rhodobacteraceae*

Kwang Kyu Kim, Jung-Sook Lee, Keun Chul Lee, Hee-Mock Oh and Song-Gun Kim

Correspondence
Song-Gun Kim
sgkim@kribb.re.kr

Korean Collection for Type Cultures, Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-806, Republic of Korea

The alphaproteobacterial strains GRP21^T and PH34, which were isolated from coastal sediment of the East Sea, Korea, were subjected to a polyphasic taxonomic investigation. The strains were Gram-negative, non-motile, non-spore-forming, oval-shaped rods that produced creamy-white colonies on tryptic soy agar, required NaCl for growth, contained Q-10 as the predominant ubiquinone, contained 16:0, 18:1 ω 7c and 19:0 cyclo ω 8c as major fatty acids and had polar lipid profiles consisting of phosphatidylcholine, phosphatidylglycerol, an unknown aminolipid, an unknown phospholipid and three unknown lipids. Phylogenetic analysis, based on 16S rRNA gene sequencing, showed that the strains were most closely related to *Donghicola eburneus* KCTC 12735^T, with 94.5 % sequence similarity, but formed a separate lineage within the family *Rhodobacteraceae*. The combined genotypic and phenotypic data supported the conclusion that the strains represent a novel genus and species, for which the name *Pontibaca methylaminivorans* gen. nov., sp. nov. is proposed. The type strain of *Pontibaca methylaminivorans* is GRP21^T (=KCTC 22497^T =DSM 21219^T).

The family *Rhodobacteraceae* (Garrrity *et al.*, 2005), which belongs to the class *Alphaproteobacteria*, contains approximately 70 recognized genera (type genus, *Rhodobacter*) at the time of writing. Nearly three-fifths of the genera in the family *Rhodobacteraceae* consist of one or two species. Over two-thirds of the species within the family originate from marine environments. Several of the genera, including *Citreimonas* (Choi & Cho, 2006), *Yangia* (Dai *et al.*, 2006), *Maritimibacter* (Lee *et al.*, 2007a), *Thalassococcus* (Lee *et al.*, 2007b), *Tateyamaria* (Kurahashi & Yokota, 2007), *Ponticoccus* (Hwang & Cho, 2008) and *Pelagicola* (Kim *et al.*, 2008), have been described in the last few years.

During a screen for trimethylamine-degrading bacteria from coastal sediments, three organisms were recovered under anaerobic conditions (Kim *et al.*, 2003). Coastal sediment samples from the East Sea, Korea, were initially enriched in mineral salts medium (MSM) supplemented with 10 mM trimethylamine and 30 mM NaNO₃ and the cultures were then serially diluted into fresh medium. Aliquots of each serial dilution were spread on MSM agar supplemented with

10 mM trimethylamine and 30 mM NaNO₃, and incubated anaerobically at 30 °C. Two creamy-white colonies were isolated and subcultured, yielding strains GRP21^T and PH34. On the basis of 16S rRNA gene sequence comparisons, the strains belong to the family *Rhodobacteraceae*, but they exhibited low levels of 16S rRNA gene sequence similarity, as calculated by the EzTaxon server (Chun *et al.*, 2007), with respect to type strains of representative species such as *Donghicola eburneus* (94.5 %), *Thalassococcus halodurans* (94.4 %), *Roseivivax halodurans* (94.3 %) and *Salipiger mucosus* (92.4 %). To elucidate the taxonomic status of the two isolates further, they were subjected to a polyphasic investigation.

For most experiments, the strains were cultivated aerobically on tryptic soy agar (Difco) containing 2 % NaCl or marine agar 2216 (Difco) at 30 °C for 3 days. For the analysis of fatty acids, the strains were cultivated on tryptic soy agar containing 5 % NaCl at 30 °C for 2 days. *Donghicola eburneus* KCTC 12735^T, *Salipiger mucosus* KCTC 22097^T, *Roseivivax halodurans* KCTC 22255^T and *Thalassococcus halodurans* KCTC 22637^T, grown under identical conditions, were used as reference strains for chemotaxonomic analysis and for other phenotypic tests.

Gram-staining was performed as described by Gerhardt *et al.* (1994). Cell morphology and motility were observed under a light microscope (Nikon Optiphot-2; $\times 1500$ magnification) with cells grown for 3–7 days. Motility was tested by the hanging-drop technique (Skerman, 1967),

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains PH34 and GRP21^T are AJ505787 and AJ505788.

A transmission electron micrograph of cells of strain GRP21^T, an extended neighbour-joining tree, results of TLC of polar lipids and fatty acid profiles at different growth phases are available as supplementary material with the online version of this paper.

and the presence of flagella was determined by transmission electron microscopy (Philips CM-20) after negative staining with 1 % (w/v) phosphotungstic acid. Oxidase activity was determined using Bactident Oxidase strips (Merck) and catalase activity was tested using 3 % H₂O₂. Growth was investigated at 5, 10, 15, 20, 25, 30, 37 and 42 °C and at pH 5–11 (in increments of 1 pH unit) using marine agar 2216 (Difco). The following biological buffers were used to adjust the pH: Na₂HPO₄/NaH₂PO₄ for pH 5–7 and Na₂CO₃/NaHCO₃ for pH 8–11 (Gomori, 1955; Bates & Bower, 1956). Growth in the absence of NaCl was investigated using tryptic soy broth prepared according to the Difco recipe with the exception that NaCl was not included. Growth at various NaCl concentrations (0.5 % and 1–15 %, at intervals of 1 %) was investigated in tryptic soy broth (Difco) or marine broth 2216 (Difco) (Yoon *et al.*, 2007). Growth on MacConkey agar (Difco) was tested, and hydrolysis of casein and starch was tested on casein agar and starch agar (both from Difco), respectively; these media were supplemented with 2 % NaCl. In order to test for the presence of bacteriochlorophyll *a*, pigments were extracted from cell pellets with acetone/methanol (7:2, v/v) in the dark and the absorption spectrum between 350 and 950 nm was determined using a Beckman Coulter DU 730 spectrophotometer (Pukall *et al.*, 1999). Tests to determine the utilization of various carbon sources, the production of acid and the physiological profiles of the two strains were performed using the API 20E, API 20NE, API 32GN, API 50CH and API ZYM galleries (bioMérieux) according to the instructions of the manufacturer.

Fatty acid methyl esters were prepared and analysed as described previously (Klatte *et al.*, 1994) using the standard Microbial Identification System (MIDI) for automated gas chromatographic analysis (Sasser, 1990; Kämpfer & Kroppenstedt, 1996). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified using TLC on Kieselgel 60 F₂₅₄ plates (20 × 20 cm, 0.5 mm thick; Merck) with petroleum ether/diethyl ether (9:1, v/v) as the solvent. The resulting bands were scraped from the plate under short-wavelength UV light, redissolved in the solvent and concentrated by nitrogen stream evaporation and the quinone profile was then analysed using reversed-phase HPLC (LC20AD system; Shimadzu) as described by Shin *et al.* (1996). Cell-wall sugars were analysed according to the procedures of Stanek & Roberts (1974). Polar lipids were extracted, examined using two-dimensional TLC and identified according to published procedures (Minnikin *et al.*, 1977). Results of TLC of polar lipids of GRP21^T and *Donghicola eburneus* KCTC 12735^T are available as Supplementary Fig. S1 in IJSEM Online.

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA gene and sequencing of purified PCR products were carried out according to Rainey *et al.* (1996) with the exception that the Prep-A-gene kit (Bio-Rad) was not used for further purification of the DNA. 16S rRNA gene sequences were aligned with published sequences

retrieved from EMBL using CLUSTAL_X (Thompson *et al.*, 1997) and were edited using BioEdit (Hall, 1999). Phylogenetic trees were constructed using sequences of approximately 1300 bp on the basis of the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. Distances were estimated by the method of Jukes & Cantor (1969) using the program MEGA version 4.0 (Kumar *et al.*, 2007). For phylogenetic analysis based on the maximum-likelihood algorithm, the PHYLIP package version 3.67 (Felsenstein, 2004) was used. The resultant tree topologies were evaluated by means of bootstrap analysis (Felsenstein, 1985) based on 1000 resampled datasets for the MEGA trees or 100 datasets for the PHYLIP trees. DNA G+C content was determined by HPLC after hydrolysis, as described by Tamaoka & Komagata (1984), and non-methylated lambda phage DNA (Sigma) was used as the standard. DNA–DNA hybridization was performed fluorometrically (Ezaki *et al.*, 1989) using DNA probes labelled with photobiotin (Sigma) in 96-well microdilution plates (Greiner Bio-One) at 50 °C.

Strains GRP21^T and PH34 formed visible colonies (approx. 0.5 mm in diameter) within 3 days on tryptic soy agar containing 2 % NaCl or marine agar 2216. Growth occurred at 15–37 °C, but no growth was observed below 10 °C or at 42 °C within 14 days. Growth occurred at pH 6–10. Cells of the two strains were Gram-negative, non-motile, facultatively anaerobic, slightly halophilic, oval-shaped rods and the colonies were creamy white. The strains were able to reduce nitrate or nitrite, allowing them to grow under anaerobic conditions. A transmission electron micrograph of cells of strain GRP21^T is available as Supplementary Fig. S2.

The almost-complete 16S rRNA gene sequences (approx. 1400 bp) of strains GRP21^T and PH34 were determined and compared with those of representative species of the genera within the family *Rhodobacteraceae*. Strains GRP21^T and PH34 shared 99.4 % 16S rRNA gene sequence similarity. They showed the highest level of similarity (94.5 %) to *Donghicola eburneus* KCTC 12735^T, as calculated by the EzTaxon server (<http://www.eztaxon.org/>). In the phylogenetic tree, strains GRP21^T and PH34 occupy a distinct position within the family *Rhodobacteraceae*, forming a branch with *Donghicola eburneus* and *Thalassococcus halodurans* (Fig. 1 and Supplementary Fig S3). DNA–DNA hybridization between strains GRP21^T and PH34 was 93 %, confirming that they belong to the same genomic species (Wayne *et al.*, 1987).

Strains GRP21^T and PH34 were clearly differentiated from related species by the presence of 19:0 cyclo ω 8c (16 %) as one of the major fatty acids. The dominant fatty acid of related taxa in the family *Rhodobacteraceae* is 18:1 ω 7c, at more than 70 %, while this fatty acid made up about 50 % of the total in strains GRP21^T and PH34 (Table 1). The fatty acid 19:0 cyclo ω 8c was absent from *Donghicola eburneus* and *Thalassococcus halodurans* but was found as a minor component in *Salipiger mucosus* and *Roseivivax halodurans* as well as being found in *Oceanicola granulosus* (10.8 %; Cho & Giovannoni, 2004), *Citireimonas salinaria* (20.4 %; Choi &

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, constructed according to the neighbour-joining method, showing the positions of strains GRP21^T and PH34 among representative species within the family *Rhodobacteraceae*. Numbers at branch points refer to bootstrap values (percentages of 1000 resamplings; only values above 50% are shown). *Stappia stellulata* IAM 12621^T was used to root the tree. Solid circles indicate branches that were found in both the maximum-likelihood and maximum-parsimony trees (bootstrap values >50%). The open circle indicates a branch point that was recovered only in the maximum-parsimony tree (bootstrap value >50%). Bar, 1 substitution per 100 nucleotide positions. An extended neighbour-joining tree including GRP21^T and almost all type species in the family *Rhodobacteraceae* is available as Supplementary Fig. S3.

Cho, 2006) and *Tateyamaria omphalii* (7.6%; Kurahashi & Yokota, 2007). The dependence of the fatty acid composition on the growth phase of strain GRP21^T was tested (Supplementary Table S1). The percentage of 19:0 cyclo ω 8c increased and that of 18:1 ω 7c decreased with length of cultivation, but

Table 1. Cellular fatty acids of strains GRP21^T and PH34 and the type strains of related species

Strains: 1, GRP21^T; 2, PH34; 3, *Donghicola eburneus* KCTC 12735^T; 4, *Thalassococcus halodurans* KCTC 22637^T; 5, *Salipiger mucosus* KCTC 22097^T; 6, *Roseivivax halodurans* KCTC 22255^T. Fatty acid compositions were analysed in this study with cells grown on tryptic soy agar for 2 days. Fatty acids are listed using standard abbreviations (number of carbon atoms:number of double bonds). Values are percentages of total fatty acids. ND, Not detected.

Fatty acid	1	2	3	4	5	6
10:0 3-OH	2.6	2.4	0.7	1.7	ND	ND
12:0	3.1	3.3	ND	ND	ND	ND
12:1 3-OH	ND	ND	3.3	5.1	5.4	ND
12:0 3-OH	3.6	3.3	ND	0.1	0.4	3.5
14:0	0.6	0.5	ND	0.1	ND	ND
Summed feature 4*	0.9	0.7	1.0	0.7	1.6	ND
16:0	15.8	14.9	6.4	7.1	16.9	3.4
17:0	0.7	0.5	ND	0.7	ND	0.6
18:1 ω 9c	ND	ND	ND	0.5	ND	ND
18:1 ω 7c	51.1	48.8	84.8	77.7	70.7	74.2
18:0	2.1	2.1	3.8	4.8	1.4	13.4
11-Methyl 18:1 ω 7c	0.9	0.8	ND	0.9	2.4	0.9
19:0 cyclo ω 8c	16.1	16.5	ND	ND	1.3	1.8

*Summed feature 4 comprises 16:1 ω 7c and/or iso-15:0 2-OH.

cells harvested at early exponential growth (day 2) already contained 19:0 cyclo ω 8c. We therefore conclude that the presence of 19:0 cyclo ω 8c is a distinct characteristic of the fatty acid compositions of GRP21^T and PH34.

The physiological, biochemical and chemotaxonomic characteristics of strains GRP21^T and PH34 are given in the species description and characteristics that serve to distinguish these strains from their closest phylogenetic neighbours, *Donghicola eburneus* and *Thalassococcus halodurans*, and related taxa are listed in Tables 1 and 2. Significant differences in terms of profiles of fatty acids, acid production, carbon source utilization and substrate hydrolysis, cell morphology, anaerobic growth and nitrite reduction support the proposal of a novel genus.

On the basis of 16S rRNA gene dissimilarity with respect to related taxa, together with the phylogenetically distinct position, unique phenotypic characteristics and genomic relatedness, strains GRP21^T and PH34 represent a novel genus and species, for which the name *Pontibaca methylaminivorans* gen. nov., sp. nov. is proposed.

Description of *Pontibaca* gen. nov.

Pontibaca [Pon.ti.ba'ca. L. n. *pontus* the sea; L. fem. n. *baca* a berry, especially an olive; N.L. fem. n. *Pontibaca* olive-shaped (bacterium) of the sea].

Cells are Gram-negative, non-motile, facultatively anaerobic, oval-shaped rods. Slightly halophilic. Bacteriochlorophyll *a* is not produced. Nitrate and nitrite are reduced. Catalase- and oxidase-positive. Major polar lipids are phosphatidylcholine and phosphatidylglycerol. Major fatty acids are 16:0, 18:1 ω 7c and 19:0 cyclo ω 8c (together representing 83% of the total in the type strain of the type species). The

Table 2. Characteristics that differentiate strains GRP21^T and PH34 from representatives of related genera

Strains: 1, GRP21^T and PH34 (*Pontibaca methylaminivorans* gen. nov., sp. nov.); 2, *Donghicola eburneus* SW-277^T; 3, *Thalassococcus halodurans* UST050418-052^T (data from Lee *et al.*, 2007b); 4, *Salipiger mucosus* A3^T; 5, *Roseivivax halodurans* Och 239^T. Data were obtained in the present study unless indicated. All strains are positive for catalase and oxidase activities and have ubiquinone Q-10 as the major quinone. +, Positive; –, negative; NA, no data available.

Characteristic	1	2	3	4	5
Morphology	Ovoid rods	Cocci or rods ^{a*}	Ovoid	Rods ^b	Rods ^c
Source	Coastal sediment	Seawater ^a	Marine sponge	Hypersaline soil ^b	Charophyte ^c
Cell length (µm)	0.9–1.6	0.6–2.0 ^a	0.7–1.0	2.0–2.5 ^b	1.0–5.0 ^c
Cell width (µm)	0.7–1.3	0.6–0.8 ^a	NA	0.75–1.0 ^b	0.5–1.0 ^c
DNA G + C content (mol%)	64.8–65.6	59.7 ^a	57.8	64.5 ^b	64.4 ^c
Growth conditions					
Anaerobic growth	+	–	–	–	–
NaCl concentration (%)					
Range	0.5–10	0.5–11	2–18	1–20	0–20
Optimum	2–3	2	2–6	9–10	5
Temperature (°C)					
Range	15–37	10–42	12–44	20–40	10–37
Optimum	30	37	28–36	37	27–30
pH					
Range	6–10	5–9	6–10	6–10	5–10
Optimum	7–8	7–8	NA	8	7.5–8
Biochemical properties					
Nitrate reduction	+	+	+	–	+
Nitrite reduction	+	–	–	–	–
Urease	+	–	–	+	–
Acid production from:					
Amygdalin	+	–	–	–	+
D-Fructose	–	+	+	–	+
Maltose	–	+	–	–	+
D-Ribose	+	–	–	–	+
Sucrose	–	+	+	–	+
D-Xylose	+	–	+	–	–
Utilization of:					
L-Arabinose	–	+	+	–	+
Gluconate	–	+	–	+	–
2-Ketogluconate	–	+	+	+	–
5-Ketogluconate	–	+	–	+	–
DL-Lactate	–	+	NA	–	+
Malate	–	+	–	+	+
D-Mannitol	–	+	+	+	+
L-Serine	+	–	NA	–	–
D-Sorbitol	–	+	+	+	–
Hydrolysis of:					
PNPG†	–	+	+	+	+
2-Naphthyl phosphate (pH 8.5)‡	–	+	+	+	+
2-Naphthyl butyrate‡	–	+	+	–	+
2-Naphthyl caprylate‡	–	+	+	–	+
2-Naphthyl phosphate (pH 5.4)‡	–	+	+	–	+
Chemotaxonomy					
Polar lipids§	PC, PG, AL, PL, L1–L3	PC, PE, PG, AL	NA	NA	NA

*Data from: a, Yoon *et al.* (2007); b, Martínez-Cánovas *et al.* (2004); c, Suzuki *et al.* (1999).

†p-Nitrophenyl β-D-galactopyranoside.

‡Data from API ZYM tests. The intensity of the colour was measured on a scale from 0 to 5 and was interpreted as negative at values of 0 or 1 and positive at values of 2–5.

§PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unknown aminolipid; PL, unknown phospholipid; L1–L3, unknown polar lipids.

predominant respiratory quinone is ubiquinone Q-10. The type species is *Pontibaca methylaminivorans*.

Description of *Pontibaca methylaminivorans* sp. nov.

Pontibaca methylaminivorans (me.thy'la.mi'ni.vo'rans. N.Gr. n. *methyl* the methyl radical; N.L. n. *aminum* the amine group; L. part. pres. *vorans* devouring; N.L. part. adj. *methylaminivorans* degrading methylated amines).

Displays the following properties in addition to those given for the genus. Cells are 0.7–1.3 × 0.9–1.6 µm. Colonies are circular, convex and creamy white. Growth occurs at 15–37 °C (optimum 30 °C), at pH 6–10 (optimum pH 7–8) and at 0.5–10 % NaCl (optimum 2–3 %). No growth is observed on MacConkey agar. Indole and H₂S are not produced. Urease is produced, but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and β-galactosidase are not. Aesculin, casein, gelatin and starch are not hydrolysed. The Voges–Proskauer test is positive. In the API 50 CH gallery, acid is produced from amygdalin, D- and L-arabinose, cellobiose, D-fucose, D-galactose, D-glucose, lactose, D-mannose, melibiose, L-rhamnose, D-ribose and D- and L-xylose, but not from N-acetylglucosamine, adonitol, D- or L-arabitol, arbutin, dulcitol, erythritol, D-fructose, L-fucose, gentiobiose, gluconate, glycerol, glycogen, *myo*-inositol, inulin, 2- or 5-ketoglucuronate, D-lyxose, maltose, D-mannitol, melezitose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-xyloside, raffinose, D-salicin, D-sorbitol, sorbose, starch, sucrose, D-tagatose, trehalose, turanose or xylitol. Acetate, 3-hydroxybutyrate and L-serine are utilized as sole carbon sources, but N-acetylglucosamine, adipate, L-alanine, L-arabinose, caprate, citrate, L-fucose, gluconate, D-glucose, glycogen, L-histidine, 3- and 4-hydroxybenzoate, *myo*-inositol, itaconate, 2- and 5-ketoglucuronate, DL-lactate, malate, malonate, maltose, D-mannitol, D-mannose, melibiose, phenylacetate, L-proline, propionate, L-rhamnose, D-ribose, D-salicin, D-sorbitol, suberate, sucrose and valerate are not. Trimethylamine, dimethylamine, methylamine and tetramethylammonium are utilized as sole carbon sources under both aerobic and anaerobic conditions. According to API ZYM tests, L-leucyl 2-naphthylamide and L-valyl 2-naphthylamide are hydrolysed, but 2-naphthyl phosphate (pH 8.5 and 5.4), 2-naphthyl butyrate, 2-naphthyl caprylate, 2-naphthyl myristate, L-cystyl 2-naphthylamide, N-benzoyl-DL-arginine 2-naphthylamide, N-glutaryl-phenylalanine 2-naphthylamide, naphthol-AS-BI-phosphate, 6-bromo-2-naphthyl α-D-galactopyranoside, 2-naphthyl β-D-galactopyranoside, naphthol-AS-BI β-D-glucuronide, 2-naphthyl α-D-glucopyranoside, 6-bromo-2-naphthyl β-D-glucopyranoside, 1-naphthyl N-acetyl-β-D-glucosaminide, 6-bromo-2-naphthyl α-D-mannopyranoside and 2-naphthyl α-L-fucopyranoside are not hydrolysed. The polar lipids are composed of phosphatidylcholine, phosphatidylglycerol, an unknown aminolipid, an unknown phospholipid and three unknown polar lipids. The cell-wall sugars are

galactose and glucose. The G + C content of the DNA is 64.8–65.6 mol% (64.8 mol% for the type strain).

The type strain is GRP21^T (=KCTC 22497^T =DSM 21219^T), isolated from a coastal sediment.

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