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TAXONOMIC DESCRIPTION

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Pseudopuniceibacterium sediminis gen. nov., sp. nov., a member of the family *Rhodobacteraceae* isolated from sediment

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Abstract

A novel Gram-stain-negative bacterium, strain CY03^T, was isolated from sediment of the Yellow Sea, PR China. Cells of strain CY03^T were rods, aerobic and non-flagellated. Growth occurred at 5–40 °C (optimum, 30 °C), pH 5.5–9.5 (pH 7.5) and with 0.5–9.0 % NaCl (1.5–2.0 %). The 16S rRNA gene sequence comparison showed affiliation to the family *Rhodobacteraceae* with *Puniceibacterium confluentis* (97.0 %) as the most closely related species, followed by members of the genus *Pseudooceanicola*, *Pseudooceanicola antarcticus* (96.8 %) and *Pseudooceanicola nitratireducens* (96.7 %). The major cellular fatty acids were cyclo-$C_{19:0}\omega8c$, $C_{16:0}$, summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and 11-methyl $C_{18:1}\omega7c$. The polar lipids consisted of phosphatidylcholine, phosphatidylglycerol, one unidentified phospholipid, one unidentified aminolipid and five unidentified lipids. The predominant respiratory quinone was Q-10. The DNA G+C content of the type strain was 62.8 mol%. Based on the results of the polyphasic characterization for strain CY03^T, it represents a novel species of a novel genus of the family *Rhodobacteraceae*, for which the name *Pseudopuniceibacterium sediminis* gen. nov., sp. nov. is proposed. The type strain is CY03^T (=CCTCC AB 2017195^T=KCTC 62198^T).

The genus *Puniceibacterium*, belonging to the family *Rhodobacteraceae* in the phylum *Proteobacteria*, was originally proposed by Liu *et al.* [1] and currently contains three species: *Puniceibacterium antarcticum* [1], *Puniceibacterium sediminis* [2] and *Puniceibacterium confluentis* [3]. Species of the genus originate from Antarctic seawater, intertidal sediment and the junction between the ocean and a freshwater spring. The genus *Pseudooceanicola* in the family *Rhodobacteraceae* was originally proposed by Lai *et al.* [4] to accommodate *Pseudooceanicola atlanticus* (type species) and six reclassified *Oceanicola* species: *Pseudooceanicola batsensis* [5], *Pseudooceanicola marinus* [6], *Pseudooceanicola nanhaiensis* [7], *Pseudooceanicola nitratireducens* [8], *Pseudooceanicola antarcticus* and *Pseudooceanicola flagellates* [9]. Species of the genus originate from sea water, with the exception of *Pseudooceanicola nanhaiensis* from marine sediments. In the course of an investigation of the cultivable bacteria diversity from marine samples, a member of the family *Rhodobacteraceae*, strain CY03^T, was isolated from sediment of the Yellow Sea, PR China. Strain CY03^T was found to be closely related to species in the genera *Puniceibacterium* and *Pseudooceanicola* based on 16S rRNA gene sequence analysis. In this study, strain CY03^T was proposed to represent a novel species in a new genus in the family *Rhodobacteraceae* based on the results of a polyphasic taxonomy study.

The surface marine sediment sample was collected at a water depth of 25 m in the Yellow Sea (37° N, 122.65° E) during a cruise in the Bohai Sea and Yellow Sea in 2014. The sample was stored in sterilized plastic bags (250 ml) at 4 °C. Strain CY03^T was isolated using the standard dilution plating technique on TYM agar [containing 0.5 % tryptone, 0.1 % yeast extract, 0.5 % skim milk, 1.5 % agar and artificial sea water (ASW) [10], pH 7.5) at 15 °C. Strain CY03^T and five reference ones [*Puniceibacterium antarcticum* SM1211^T, *Pseudooceanicola antarcticus* CGMCC 1.12662^T (purchased from CGMCC), *Pseudooceanicola nitratireducens* CGMCC 1.7292^T (purchased from CGMCC), *Pseudooceanicola marinus* LMG 23705^T (purchased from MCCC) and *Pseudooceanicola atlanticus* MCCC 1A09160^T (purchased from MCCC)] were all routinely cultivated in marine broth 2216

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Abbreviations: ASW, artificial sea water; FAME, fatty acid methyl ester; MA, marine agar 2216; MB, marine broth 2216; Q-10, ubiquinone 10.

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The GenBank/EMBL/DDBJ accession numbers for the genome sequence and the 16S rRNA gene sequence of strain CY03^T are QWJJ00000000 and KY777998, respectively.

Two supplementary tables and two supplementary figures are available with the online version of this article.

(MB; Difco) or on marine agar 2216 (MA; MB with 1.5 % agar) at 30 °C and preserved in MB containing glycerol (20 %, v/v) at −80 °C.

Genomic DNA was extracted using an EZNA bacterial DNA kit (Omega) following the manufacturer's instructions except that sterile double distilled H_2O was used for DNA elution. The 16S rRNA gene was amplified from the extracted genomic DNA by PCR with the universal bacterial primers 27F and 1492R [11]. The PCR product was purified using the GeneJET gel extraction kit (Thermo) and then ligated into pMD 19-T vector (Tsingke) and sequenced using an automated DNA sequencer (model 3730xl, Applied Biosystems) at Tsingke Biological Technology Co. Ltd (Qingdao, PR China). The obtained 16S rRNA gene sequence of strain CY03^T was compared to those of species validly published through the EzBioCloud server [12]. Phylogenetic trees were reconstructed with MEGA 6 [13] using the maximum-likelihood [14], neighbour-joining [15] and minimum-evolution [16] methods. The evolutionary distances were computed with Kimura's two-parameter model [17]. The topologies of the phylogenetic trees were evaluated by bootstrap analyses (1000 replications). Genome sequencing was performed at BGI-Shenzhen (PR China) using the HiSeq 4000 sequencer system (Illumina). The DNA G+C content was calculated directly from the draft genome sequences.

A nearly full-length (1425 bp) 16S rRNA gene sequence of CY03^T was obtained. Strain CY03^T showed the highest 16S rRNA gene sequence similarities to *Puniceibacterium confluentis* (97.0 %), followed by *Pseudooceanicola antarcticus* (96.8 %) and *Pseudooceanicola nitratireducens* (96.7 %). Strain CY03^T also shared relatively high sequence similarities (≥96.0 but ≤96.5 %) with the type strains of the following species in the family *Rhodobacteraceae*: *Puniceibacterium antarcticum* (96.5 %), *Puniceibacterium sediminis* (96.3 %), *Pseudooceanicola marinus* (96.2 %), *Citreicella aestuarii* (96.2 %), *Phaeobacter porticola* (96.1 %), *Phaeobacter gallaeciensis* (96.0 %), *Phaeobacter inhibens* (96.0 %) and *Marivita lacus* (96.0 %). However, similarities to the type strains of other recognized species in the family *Rhodobacteraceae* were all below 96.0 %. In the maximum-likelihood tree, strain CY03^T formed a coherent clade with type species of the genus *Puniceibacterium*. Two other forms of phylogenetic trees generated a similar tree topology (Fig. 1). The genomic DNA G+C content of strain CY03^T was determined to be 62.8 mol%, which was within the range of G+C contents reported for the genus *Puniceibacterium* (59.1–64.4 %) and *Pseudooceanicola* (61.8–72.8 mol%).

The respiratory quinone of strain CY03^T was extracted, separated and analysed as described by Lin *et al.* [18]. The fatty acid methyl esters (FAMEs) of strain CY03^T were analysed by using an Agilent 6890N gas chromatograph and identified using the Sherlock Microbial Identification System (MIDI; version 4.5 and the TSBA40 database) at the Shanghai Public Health Clinical Centre after cultivation in TYS broth [19] at 28 °C for 36 h. Furthermore, cells mass of CY03^T and reference strains (*Puniceibacterium antarcticum* SM1211^T, *Pseudooceanicola antarcticus* CGMCC 1.12662^T, *Pseudooceanicola nitratireducens* CGMCC 1.7292^T, *Pseudooceanicola marinus* LMG 23705^T and *Pseudooceanicola atlanticus* MCCC 1A09160^T) were collected from the third quadrant of the quadrant streaked plate (at exponential phase) after incubated on MA medium at 28 °C. FAMEs were also analysed by gas chromatograph (Agilent 6850N) using the Sherlock Microbial Identification System (TSBA, version 6.1, MIDI) at the Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, PR China. Polar lipids of strain CY03^T and reference strains were extracted according to Komagata and Suzuki [20] and analysed using two-dimensional TLC on silica gel 60 F254 plates (Merck) with appropriate spraying reagents including ethanolic molybdophosphoric acid (total lipids), ninhydrin (aminolipids), molybdenum blue (phospholipids) and a-naphthol (glycolipids).

The predominant respiratory quinone of CY03^T was Q-10, which is typical for the majority of the class *Alphaproteobacteria*. Cellular fatty acid comparisons of strain CY03^{T}and five reference ones are shown in Table S1 (available in online version of this article). Though the proportion of fatty acids was affected by culture conditions, the major cellular fatty acids (>8.0 % of the total fatty acids) of strain CY03^T in different culture conditions (in TYS broth at 28 °C for 36 h versus on MA medium at 28 °C) were cyclo-$C_{19:0}$ $\omega8c$, $C_{16:0}$, summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and 11-methyl $C_{18:1}\omega7c$. Under the same cultural conditions, the fatty acid pattern of strain CY03^T was distinguishable from that of *Puniceibacterium antarcticum* SM1211^T in the proportions of summed feature 8 (17.1 % versus 61.0 %), cyclo-$C_{19:0}\omega8c$ (35.4 % versus trace amount) and 11-methyl $C_{18:1}\omega7c$ (9.0 % versus 1.9 %), and from that of *Pseudooceanicola nitratireducens* CGMCC 1.7292^T in the proportions of cyclo-$C_{19:0}\omega8c$ (35.4 % versus not detected), and from that of *Pseudooceanicola atlanticus* MCCC 1A09160^T in the proportions of cyclo-$C_{19:0}\omega8c$ (35.4 % versus 12.1 %), summed feature 8 (17.1 % versus 59.7 %) and 11-methyl $C_{18:1}\omega7c$ (9.0 % versus 1.2 %), and also from that of *Pseudooceanicola antarcticus* CGMCC 1.12662^T and *Pseudooceanicola marinus* LMG 23705^T in the proportion of $C_{12:1}$ 3OH (not detected versus 7.6 versus 4.0). The predominant cellular fatty acid of the genus *Puniceibacterium* is $C_{18:1}\omega7c$ [1–3], the major fatty acids of the genus *Pseudooceanicola* include summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), 11-methyl $C_{18:1}\omega7c$, cyclo-$C_{19:0}\omega8c$, $C_{12:0}$ 3OH or $C_{16:0}$ [21]. The polar lipids of strain CY03^T mainly included phosphatidylcholine, phosphatidylglycerol, one unidentified phospholipid, one unidentified aminolipid and five unidentified lipids. Phosphatidylethanolamine was not detected in strain CY03^T while it was one of the major polar lipids in the reference species *Puniceibacterium antarcticum* SM1211^T, *Pseudooceanicola nitratireducens* CGMCC 1.7292^T, *Pseudooceanicola marinus* LMG 23705^T and *Pseudooceanicola atlanticus* MCCC 1A09160^T (Fig. S1). Thus, strain CY03^T

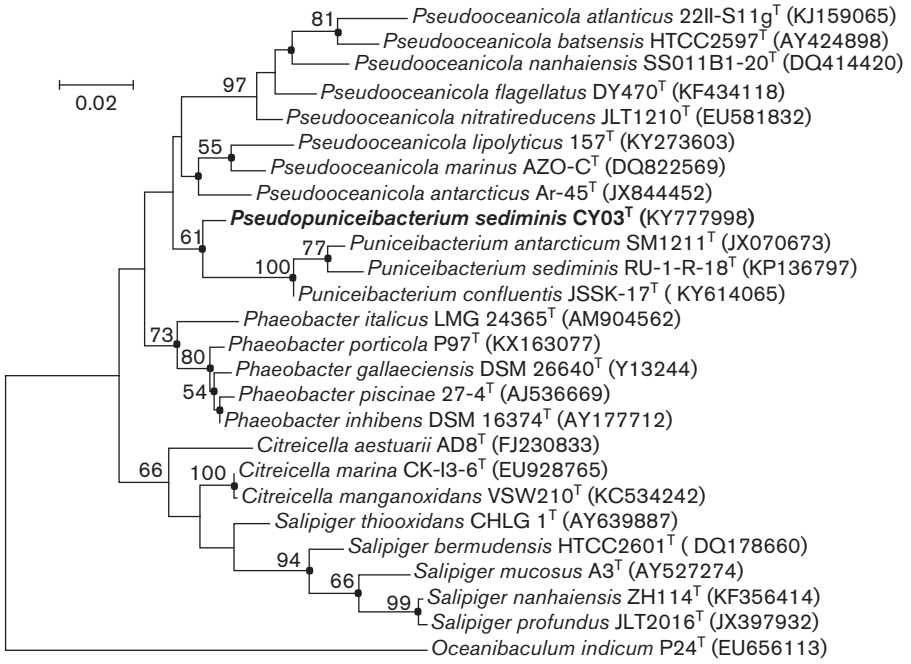

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences of *Pseudopuniceibacterium sediminis* CY03^T (in bold) and representatives of closely related species. The tree was considered 1302 nucleotide positions and calculated based in 1000 replications. Bootstrap values (>50 %) are shown at the branching points. Filled circles indicate that the corresponding nodes were also recovered in the neighbour-joining and minimum-evolution trees for the same sequences. Bar, 0.02 substitutions per nucleotide position. *Oceanibaculum indicum* P24^T was used as outgroup.

can be distinguished from closely related species of the genera *Puniceibacterium* and *Pseudooceanicola*.

Cell morphology was observed using light microscope (CX31, Olympus) and transmission electron microscope (HT7700, Hitachi) with cells from exponentially growing cultures. Gram staining was carried out according to Murray *et al.* [22]. Colony morphology was observed after incubation on MA at 30 °C for 2 days. The catalase and oxidase activities were tested with 3 % (v/v) H_2O_2 and oxidase reagent (Hopebiol Microorganism Reagent), respectively. Growth at temperatures (5, 10, 15, 20, 25, 30, 37, 40 and 45 °C) was measured in MB. Growth with different NaCl concentrations (0–5.0 %, at intervals of 0.5 % units; 6.0–10.0 %, at intervals of 1.0 % units; w/v) was determined in NP broth (containing 0.5 % tryptone, 0.1 % yeast extract, 0.5 % $MgCl_2$, 0.2 % $MgSO_4$, 0.1 % $CaCl_2$, 0.1 % KCl and distilled water, pH 7.05) [19]. Growth at pH 5–10.0 [at intervals of 0.5 pH units, buffed with MES (pH 5.0–6.0, 50 mM), MOPS (pH 6.5–7.0, 50 mM), Tris (pH 7.5–8.5, 50 mM) and CHES (pH 9.0–10.0, 50 mM)] were assessed in NP broth with 2.0 % NaCl. Degradation of starch (0.2 %, w/v), casein (0.5 %, w/v), Tween 20, Tween 40, Tween 80, xanthine and hypoxanthin (1 %, w/v) was examined by using MA as the basal medium and incubation at 30 °C for 5 days. Antibiotic resistance was examined with the disc-diffusion method. Growth under the anaerobic condition was determined in MB supplemented with potassium nitrate (0.1 %, w/v), cysteine hydrochloride (0.05 %, w/v) and sodium sulfide (0.05 %, w/v) in Hungate tubes filled with oxygen-free N_2 at 30 °C for 2 weeks. Acid production from carbohydrates was tested at 30 °C for 10 days using modified O/F medium (0.1 % tryptone, 0.01 % yeast extract, 0.05 % $(NH_4)_2SO_4$, 0.05 % Tris, 0.001 % phenol red, 1 % carbohydrate, 0.3 % agar and half-strength artificial seawater; [23]). The utilization of carbohydrates (0.2 %, w/v) as sole carbon and energy sources was determined according to Huang *et al.* [21] at 30 °C for 20 days. Other biochemical characteristics were determined using API ZYM (ASW as the cell suspension solution), API 20NE (ASW as the cell suspension solution), API 50 CH microtest systems (bioMerieux) and the Biolog GEN III system (inoculation fluid B for the cell suspension) following the manufacturers' instructions.

Cells of strain CY03^T were found to be Gram-stain-negative, aerobic, non-flagellated, rods, 0.8–1.3 μm wide and 1.4–2.2 μm long (Fig. S2). They were sensitive to amoxycillin (10 μg), ampicillin (10 μg), bacitracin (10 U), carbenicillin (100 μg), cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), neomycin (30 μg), nitrofurantoin (300 μg), penicillin G (10 U) and rifampicin (5 μg). The detailed

Table 1. Differential characteristics of strain CY03^T and the type strains of closely related species

Strains: 1, CY03^T; 2, *Puniceibacterium antarcticum* SM1211^T; 3, *Pseudooceanicola antarcticus* CGMCC 1.12662^T; 4, *Pseudooceanicola nitratireducens* CGMCC 1.7292^T; 5, *Pseudooceanicola marinus* LMG 23705^T; 6, *Pseudooceanicola atlanticus* MCCC 1A09160^T; All data are from this study, except for morphological properties and DNA G+C contents of the reference strains, which are taken from Liu *et al.* [1], Huo *et al.* [9], Zheng *et al.* [24], Lin *et al.* [25] and Lai *et al.* [4]. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6
Cell size (μm):						
Width	0.8–1.3	0.5–0.8	0.5–0.8	0.4–1.1	0.5	0.8–1.1
Length	1.4–2.2	0.8–1.3	0.8–1.3	0.7–2.1	0.9–1.0	2.2–2.7
Flagella	–	–	–	Polar or subpolar	–	–
Growth at/with:						
40 °C	w	–	+	–	+	–
10 % (w/v) NaCl	–	+	+	–	–	–
Hydrolysis of:						
Tween 80	–	–	–	+	–	–
Enzyme activity (API ZYM):						
Trypsin	–	–	+	–	–	–
α-Chymotrypsin	–	–	+	–	–	–
α-Glucosidase	+	+	+	–	+	–
N-Acetyl-β-glucosaminidase	–	–	+	–	–	–
API 20 NE results:						
Urease	+	–	+	–	+	+
β-Glucosidase	–	–	+	–	–	–
β-Galactosidase	–	–	+	–	+	–
Assimilation of mannitol	+	+	–	–	+	–
Assimilation of maltose	+	+	–	–	–	–
Assimilation of adipate	+	–	–	–	–	–
Assimilation of malate	+	+	–	–	–	–
Assimilation of citrate	–	+	–	+	–	–
DNA G+C content (mol%)	62.8	60.7	62.0	72.8	70.9	64.1
Isolation source	Marine sediment	Antarctic seawater	Seawater	Seawater	Seawater	Seawater

results of physiological and biochemical analyses are mentioned in Tables 1 and S2 and the species description.

Strain CY03^T could be distinguished from *Puniceibacterium antarcticum* SM1211^T by the ability to grow at 40 °C, the optimum temperature for growth, the colony colour, urease, DNA G+C content and inability to grow with 10.0 % (w/v) NaCl. Especially, significant differences between the content of polar lipids and fatty acid profiles of strain CY03^T and the genus *Puniceibacterium* as described above. Due to its distinctive features (such as the phylogenetic position based on the 16S rRNA gene sequence, physiological and biochemical differences as well as clear differences in the polar lipid and the fatty acid composition compared with closely related genera *Puniceibacterium* and *Pseudooceanicola*), strain CY03^T represents a novel genus and species in the family *Rhodobacteraceae*, for which the name *Pseudopuniceibacterium sediminis* gen. nov., sp. nov. is proposed.

DESCRIPTION OF *PSEUDOPUNICEIBACTERIUM* GEN. NOV.

Pseudopuniceibacterium (Pseu.do. pu.ni.ce.i.bac.te'ri.um. Gr. adj. pseudês false; N.L. masc. n. *Puniceibacterium* a bacterial genus; N.L. masc. n. *Pseudopuniceibacterium* the false *Puniceibacterium*, referring to the close relationship to the genus *Puniceibacterium)*.

Cells are Gram-stain-negative. The major cellular fatty acids are cyclo-$C_{19:0}\omega 8c$, $C_{16:0}$, summed feature 8 ($C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$) and 11-methyl $C_{18:1}\omega 7c$. The predominant lipids are phosphatidylcholine, phosphatidylglycerol, one unidentified phospholipid and one unidentified aminolipid. The predominant respiratory quinone is Q-10. Phylogenetically, the genus *Pseudopuniceibacterium* is affiliated with the family *Rhodobacteraceae* of the class *Alphaproteobacteria*. The type species is *Pseudopuniceibacterium sediminis*.

DESCRIPTION OF *PSEUDOPUNICEIBACTERIUM SEDIMINIS* SP. NOV.

Pseudopuniceibacterium sediminis (se.di′mi.nis. L. gen. n. *sediminis* of sediment).

Cells are Gram-stain-negative, aerobic, non-flagellated, rods, approximately 0.8–1.3 μm wide and 1.4–2.2 μm long. Colonies are circular with regular edges, opaque, slightly convex and approximately 0.6–1.2 mm in diameter on MA after 48 h incubation at 30 °C. Growth occurs at 5–40 °C (optimum, 30 °C) and in pH 5.5–9.5 (pH 7.5). Grows with 0.5–9 % NaCl (1.5–2 %). Positive for catalase and oxidase, but negative for hydrolysis of casein, skimmed milk, starch, Tween 20, Tween 40, Tween 80, xanthine and hypoxanthine. Acids are produced in modified O/F medium from D-glucose, D-xylose, L-sorbose, D-mannitol, D-galactose, L-arabinose, cellobiose and D-mannose in both aerobic and anaerobic conditions. Able to utilize D-glucose, sucrose, D-xylose, cellobiose, L-arabinose, L-rhamnose, D-galactose, D-mannose, D-mannitol and sorbitol, but D-fructose, inulin, maltose monohydrate, inositol, trehalose, raffinose pentahydrate and xylitol are not utilized. The major cellular fatty acids are cyclo-$C_{19:0}\omega8c$, $C_{16:0}$, summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and 11-methyl $C_{18:1}\omega7c$. The polar lipids consist of phosphatidylcholine, phosphatidylglycerol, one unidentified phospholipid, one unidentified aminolipid and five unidentified lipids. The predominant respiratory quinone is Q-10.

The type strain is CY03^T (=CCTCC AB 2017195^T=KCTC 62198^T), isolated from sediment of the Yellow Sea. The DNA G+C content of the type strain is 62.8 mol%.

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

Ethical statement
No experiments with humans or animals were carried out.

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