Ponticoccus litoralis gen. nov., sp. nov., a marine bacterium in the family *Rhodobacteraceae*

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A Gram-negative, coccoid to rod-shaped, strictly aerobic bacterium, strain CL-GR66^T, was isolated from coastal seawater collected off Korea. The strain grew optimally in the presence of 3–5 % sea salts, at a temperature of 30 °C and at pH 7. The polar lipid profile of strain CL-GR66^T comprised phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, two unidentified aminolipids, an unidentified glycolipid and an unidentified lipid. The major fatty acids were C_{18:1}ω7c (71.4 %), 11-methyl C_{18:1}ω7c (11.3 %), C_{16:0} (6.4 %), C_{18:0} (4.6 %) and C_{12:1} 3-OH (3.0 %). Ubiquinone 10 was the major quinone. The DNA G + C content was 67.9 mol%. 16S rRNA gene sequence analysis revealed that strain CL-GR66^T belonged to the *Roseobacter* clade within the family *Rhodobacteraceae*. Strain CL-GR66^T was related most closely to the type strain of *Marinovum algicola* (95.5 % 16S rRNA gene sequence similarity), and sequence similarities between strain CL-GR66^T and other type species of the *Roseobacter* clade ranged from 91.8 to 95.4 %. Phylogenetic analyses of 16S rRNA gene sequences showed that strain CL-GR66^T was not associated with any known genus in the family *Rhodobacteraceae*. The distinct phylogenetic position of strain CL-GR66^T, its phylogenetic distance from genera of the family *Rhodobacteraceae*, together with phenotypic data, suggest that the organism represents a novel species of a new genus, for which the name *Ponticoccus litoralis* gen. nov., sp. nov. is proposed. The type strain of *Ponticoccus litoralis* is CL-GR66^T (=KCCM 90028^T =DSM 18986^T).

The *Roseobacter* clade, which was named after the genus *Roseobacter* by Giovannoni & Rappé (2000), is classified within the family *Rhodobacteraceae*, which in turn belongs to subgroup 3 of the *Alphaproteobacteria* (Woese *et al.*, 1984). At the time of writing, this clade contains more than 35 genera (Buchan *et al.*, 2005). Bacterial species affiliated with the *Roseobacter* clade have been isolated from various marine environments, such as seawater, sediment, marine algae, hypersaline microbial mats and marine biofilms (Buchan *et al.*, 2005; Choi & Cho, 2006a; Dai *et al.*, 2006). Their physiological properties are highly diverse, including aerobic anoxygenic photosynthesis, aerobic sulfite oxidation, organic sulfur compound degradation, lignin degradation, methylotrophy and antibiotic production (Buchan *et al.*, 2005; Martens *et al.*, 2006). Here we

describe a bacterial strain isolated from coastal seawater that was characterized based on a polyphasic approach (Vandamme *et al.*, 1996). On the basis of its chemotaxonomic, physiological and phylogenetic characteristics, we propose that this bacterium represents a novel species of a new genus in the family *Rhodobacteraceae*.

A coastal water sample was incubated with sand sediment in a 150 mm-diameter glass Petri dish at room temperature for approximately 15 months. Without disturbing the sediment, 100 µl surficial seawater was taken and spread on a marine agar 2216 (MA; Difco) plate, which was then incubated at 30 °C for 1 week. Strain CL-GR66^T was isolated and subsequently purified four times on MA at 30 °C. The strain was maintained both on MA at 30 °C and in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

Morphological and physiological tests were performed as follows. Gram staining was performed according to Smibert & Krieg (1994). Cell motility was observed via the hanging-drop method (Suzuki *et al.*, 2001). Cell morphology and the presence of flagella were observed by transmission electron microscopy (EX2; JEOL). Anaerobic growth was checked on MA and ZOF medium (Lemos *et al.*, 1985) supplemented with additional agar

Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CL-GR66^T is EF211829.

A transmission electron micrograph of negatively stained cells, two-dimensional TLCs of polar lipids, an extended neighbour-joining tree derived from 16S rRNA gene sequences and a table detailing the cellular fatty acid composition of strain CL-GR66^T and phylogenetically related genera in the family *Rhodobacteraceae* are available as supplementary material with the online version of this paper.

(1.5 %) by using the GasPak anaerobic system (BBL) at 30 °C for 15 days. Poly- β -hydroxybutyrate (PHB) granules were observed by epifluorescence microscopy (BX60; Olympus) after Nile blue A staining (Ostle & Holt, 1982). Bacteriochlorophyll *a* production was determined in 90 % acetone extracts with a spectrophotometer (Ultraspec 2000; Pharmacia Biotech) for cells that had been grown in either the light or the dark for 7 days. The presence of photosynthetic reaction-centre genes *pufL* and *pufM* was determined by using PCR amplification with gene-specific primers (Allgaier *et al.*, 2003) for strain CL-GR66^T, with *Porphyrobacter donghaensis* SW-132^T (=KCTC 12229^T; Yoon *et al.*, 2004) as a positive control.

The temperature range for growth was examined on the basis of colony formation on MA incubated at temperatures ranging from 5 to 40 °C, in increments of 5 °C. The pH range (5–10, in increments of 1 pH unit) for growth was determined by assessing changes in OD₆₀₀ over the incubation period (up to 7 days) in MB at 30 °C. The final pH was adjusted by using 1 M NaOH and 1 M HCl. Tolerance of strain CL-GR66^T to sea salts (Sigma) or NaCl was determined by assessing changes in OD₆₀₀ in Zobell broth (5 g Bacto peptone, 1 g yeast extract, 0.1 g ferric citrate, 1 l distilled water) at sea salts or NaCl concentrations (0–10 % in increments of 1 %, and 15 %, w/v) at 30 °C.

Oxidase and catalase tests were performed according to the protocols described by Smibert & Krieg (1994). Gelatinase, amylase and nitrate reductase activities and degradation of Tween 80 were determined according to Hansen & Sørheim (1991). Other enzyme activities were assayed by using the API ZYM kit (bioMérieux) according to the manufacturer's instructions, except that the cell suspension was prepared by using artificial seawater (24 g NaCl, 5.1 g MgCl₂, 4 g Na₂SO₄, 1.1 g CaCl₂, 0.7 g KCl, 0.2 g NaHCO₃, 0.1 g KBr, 0.027 g H₃BO₃, 0.024 g SrCl₂, 0.003 g NaF, 1 l distilled water; Lyman & Fleming, 1940). Carbon utilization was tested by using basal broth medium supplemented with yeast extract (23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O, 1.3 g CaCl₂·2H₂O, 0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract, 1 l distilled water; Bruns *et al.*, 2001) containing 0.4 % carbon source. Strain CL-GR66^T was incubated for 4 weeks and carbon utilization was scored as negative when the growth rate was equal to or less than that in the negative control with no carbon source. Growth rate was measured by monitoring changes in OD₆₀₀. Resistance to antibiotics was determined by the disc-diffusion plate method (Bauer *et al.*, 1966).

Polar lipids were extracted by using the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Fatty acid methyl esters in whole cells of strain CL-GR66^T grown on MA at 30 °C for 4 days were analysed by GC according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center

of Microorganisms (KCCM), Seoul, Korea. The quinone system was determined according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985). The DNA G+C content was analysed by HPLC (HP 100; Hewlett Packard) analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989), after DNA extraction according to the method of Marmur (1961). Lambda DNA was used as a standard.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony via a boiling method (Englen & Kelley, 2000). The crude extracts served as template DNA. *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991) were used for PCR experiments. The PCR product was purified by using the AccuPrep PCR purification kit (Bioneer) and direct sequence determination of the purified 16S rRNA gene was performed with an Applied Biosystems automated sequencer (ABI3730XL) at Macrogen, Seoul, Korea. The almost-complete 16S rRNA gene sequence of strain CL-GR66^T (1357 nt) was obtained and compared with available 16S rRNA gene sequences in GenBank by using BLASTN searches (Altschul *et al.*, 1990). The sequence of strain CL-GR66^T was manually aligned with those of species in the family *Rhodobacteraceae*, obtained from the GenBank and Ribosomal Database Project (Cole *et al.*, 2003) databases, using known 16S rRNA secondary-structure information. Phylogenetic trees were obtained by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of tree topologies was assessed by bootstrap analyses based on 1000 replications for the neighbour-joining and maximum-parsimony methods. Alignment analysis was carried out by using the jPHYDIT program (Jeon *et al.*, 2005), and phylogenetic analyses were carried out by using MEGA 3 (Kumar *et al.*, 2004).

Cells of strain CL-GR66^T were Gram-negative, coccoid to rod-shaped and approximately 0.5–0.8 µm wide and 0.8–1.2 µm long (see Supplementary Fig. S1 in IJSEM Online). The cells were non-motile. After 3 days on MA at 30 °C, colonies were creamy and approximately 2 mm in diameter. Strain CL-GR66^T was strictly aerobic (Table 1) and contained PHB granules. Bacteriochlorophyll *a* and the *pufL* and *pufM* genes were not detected. Other phenotypic characteristics of strain CL-GR66^T are given in the genus and species descriptions and in Table 1.

The polar lipid profile of strain CL-GR66^T comprised phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified glycolipid, two unidentified aminolipids and an unidentified lipid (Supplementary Fig. S2). The predominant fatty acid of strain CL-GR66^T was C_{18:1}ω7c (71.4 %), which is a feature of the vast majority of species within the *Alphaproteobacteria* (Martens *et al.*, 2006), followed by 11-methyl C_{18:1}ω7c (11.3 %), C_{16:0} (6.4 %), C_{18:0} (4.6 %) and C_{12:1} 3-OH (3.0 %).

Table 1. Selected characteristics that differentiate strain CL-GR66^T from related genera in the family *Rhodobacteraceae*

Genera: 1, *Ponticoccus* gen. nov. (strain CL-GR66^T); 2, *Marinovum* (data from Lafay *et al.*, 1995; Labrenz *et al.*, 2000; Martens *et al.*, 2006); 3, *Pelagibaca* (Cho & Giovannoni, 2006); 4, *Citricella* (Sorokin *et al.*, 2005); 5, *Thalassobius* (Arahal *et al.*, 2005; Macián *et al.*, 2005; Yi & Chun, 2006); 6, *Sagittula* (Gonzalez *et al.*, 1997); 7, *Antarctobacter* (Labrenz *et al.*, 1998, 2000); 8, *Salipiger* (Martínez-Cánovas *et al.*, 2004); 9, *Roseivivax* (Suzuki *et al.*, 1999); 10, *Donghicola* (Yoon *et al.*, 2007a); 11, *Citreimonas* (Choi & Cho, 2006b); 12, *Leisingera* (Schaefer *et al.*, 2002); 13, *Ruegeria* (Staley *et al.*, 2005; Muramatsu *et al.*, 2007); 14, *Silicibacter* (Petursdottir & Kristjansson, 1997; González *et al.*, 2003); 15, *Roseovarius* (Labrenz *et al.*, 1999; González *et al.*, 2003; Boettcher *et al.*, 2005; Biebl *et al.*, 2005); 16, *Yangia* (Dai *et al.*, 2006); 17, *Shimia* (Choi & Cho, 2006a); 18, *Phaeobacter* (Martens *et al.*, 2006; Yoon *et al.*, 2007b). +, Positive; w, weakly positive; −, negative; v, variable; ND, no data available; BChl, bacteriochlorophyll.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Species (<i>n</i>)	1	1	1	1	3	1	1	1	2	1	1	1	2	2	4	1	1	3
Cell morphology*	C–R	O–R	SR	O–SR	O–IR	R	R	R	R	C–R	OR	R–OR	R–OR	R	R–O	R	R	OR
Colony colour†	C	BG, ~P	C	WH	SP or C	C	BRY	C	P	I	BG	–	BG	BG	C, BG–PBG, R	FY	BG	BR, –
Anaerobic growth	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Motility	–	+	–	–	V	–	V	–	+	–	–	+	V	+	V	+	+	+
Growth at:																		
4 °C	–	–	–	–	V	–	+	–	ND	–	–	–	–	–	V	–	–	V
37 °C	+	+	+	–	+	+	+	+	ND	+	+	–	–	+	+	+	–	V
Nitrate reduction	+	–	+	–	V	–	+	–	V	+	+	–	+	–	V	–	+	V
Hydrolysis of:																		
Starch	+	w	ND	ND	–	–	V	–	–	–	–	+	–	–	–	–	+	–
Gelatin	+	+	+	–‡	V	–	+	–	V	–	+	ND	–	+	–	–	+	–
PHB	+	–	–	+	+	+	+	+	ND	ND	+	ND	–	–	ND	+	–	ND
BChl <i>a</i>	–	–	–	–	+	–	–	–	+	–	–	ND	–	–	V	–	–	+
Utilization of:																		
Succinate	–	–	+	+	ND	+	+	–	+	+	+	ND	+	+	+	ND	+	+
Acetate	+	+	+	+	+	ND	+	–	+	+	+	–	ND	+	+	ND	–	V
D-Glucose	+	+	+	+	+	+	+	–	+	+	+	–	+	V	+	–	–	+
DNA G + C content (mol%)	67.9	60–65	65.4	67.5–69.2	57–61	65.0	62–64	64.5	59.7–64.4	59.7	67.3	60.5	55–58.7	66.2–68	59–66	63.3	57.2	55.7–64.9

*C, Cocci; O, ovoid; R, rods; IR, irregular rods; OR, ovoid rods; SR, short rods.

†BG, Beige; BR, brown; BRY, brownish yellow; C, cream; FY, faint yellowish; I, ivory; P, pink; ~P, pinkish; PBG, pinkish beige; R, red; SP, salmon pink; WH, white; –, non-pigmented.

‡Data from Choi & Cho (2006b).

Fig. 1. Neighbour-joining tree derived from 16S rRNA gene sequences showing the phylogenetic position of strain CL-GR66^T and related members in the family *Rhodobacteraceae*. Bootstrap values (based on 1000 resamplings) are given at branch points; only values >60% are shown. Solid circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. *Alteromonas marina* SW-47^T was used as an outgroup. Bar, 0.02 nucleotide substitutions per site.

(Supplementary Table S1). The major isoprenoid quinone was ubiquinone 10. The DNA G+C content was 67.9 mol%.

Analysis of the 16S rRNA gene sequence of strain CL-GR66^T revealed a clear affiliation with the *Roseobacter* clade in the family *Rhodobacteraceae* (Fig. 1). Strain CL-GR66^T was related most closely to the type strains of *Marinovum algicola* (95.5% 16S rRNA gene sequence similarity), *Pelagibaca bermudensis* (95.4%), *Citricella thiooxidans* (95.3%), *Oceanicola batsensis* (95.1%), *Thalassobius mediterraneus* (95.1%) and *Sagittula stellata* (95.0%); levels of 16S rRNA gene sequence similarity to the type strains of other type species of the *Roseobacter* clade were between 91.8 and 94.9%. In spite of a rather high level of sequence similarity (>95%) between strain CL-GR66^T and the type strains of the above species, strain CL-GR66^T did not form a robust clade with these or any other species in the *Roseobacter* lineage. In the phylogenetic tree including other genera in the family *Rhodobacteraceae*, strain CL-GR66^T was weakly related to *Antarctobacter* and *Sagittula* (Fig. 1). Again, the cluster comprising the genera *Antarctobacter* and *Sagittula* plus strain CL-GR66^T was

not robustly supported in the phylogenetic tree in which most species in the family *Rhodobacteraceae* were included (Supplementary Fig. S3). Thus, strain CL-GR66^T formed a distinct lineage in all 16S rRNA gene sequence-based phylogenetic trees (Fig. 1, Supplementary Fig. S3). Given mean sequence divergence values of >4.1% (SD 1.6%; data not shown) among established genera of the *Roseobacter* clade, the observed sequence divergence of >4.5% between strain CL-GR66^T and genera of this clade suggests that the lineage represented by strain CL-GR66^T is equivalent in rank to the genera of the *Roseobacter* clade.

In addition, several phenotypic and chemotaxonomic characteristics can be used to differentiate strain CL-GR66^T from related genera in the family *Rhodobacteraceae*. Strain CL-GR66^T can be differentiated from members of the genus *Antarctobacter* based on the presence of 11-methyl $C_{18:1\omega7c}$ (11.3%; Supplementary Table S1) and the presence of phosphatidylethanolamine and an additional aminolipid (Supplementary Fig. S2; Labrenz *et al.*, 1998). These taxa are also distinguished by the ability to grow at 4 °C, different optimal temperature for growth (i.e. 30 and 16–26 °C for strain CL-GR66^T and *Antarctobacter*

heliothermus, respectively) and utilization of succinate and L-rhamnose (Table 1; Labrenz *et al.*, 1998). Strain CL-GR66^T is distinguishable from members of the genus *Marinovum*, including its closest phylogenetic relative, based on motility, the ability to reduce nitrate, PHB production and utilization of L-arabinose, *myo*-inositol, L-lysine, D-mannitol and D-ribose (Table 1; Lafay *et al.*, 1995). An additional aminolipid was uniquely detected in strain CL-GR66^T, although phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and an unidentified aminolipid are commonly found in members of the genus *Marinovum* (Supplementary Fig. S2; Martens *et al.*, 2006). In addition, combinations of phenotypic and chemotaxonomic characteristics can be used to differentiate strain CL-GR66^T from other related genera in the family *Rhodobacteraceae* (Table 1, Supplementary Table S1). The results of the present polyphasic study therefore indicate that strain CL-GR66^T represents a novel species of a new genus, for which the name *Ponticoccus litoralis* gen. nov., sp. nov. is proposed.

Description of *Ponticoccus* gen. nov.

Ponticoccus (Pon.ti.coc'cus. L. n. *pontus* the sea; N.L. masc. n. *coccus* berry; N.L. masc. n. *Ponticoccus coccus* from the sea).

Cells are Gram-negative, coccoid to rod-shaped. Non-motile. Growth is strictly aerobic. Oxidase- and catalase-positive. Bacteriochlorophyll *a* is not detected. Polar lipids are phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, an unidentified glycolipid, two unidentified aminolipids and an unidentified lipid. Dominant cellular fatty acids are C_{18:1}ω7c, 11-methyl C_{18:1}ω7c, C_{16:0}, C_{18:0} and C_{12:1} 3-OH. The isoprenoid quinone is Q-10. The DNA G + C content of the type strain of the type species is 67.9 mol%. Phylogenetically, the genus is a member of the family *Rhodobacteraceae*. The type species is *Ponticoccus litoralis*.

Description of *Ponticoccus litoralis* sp. nov.

Ponticoccus litoralis (li.to.ra'lis. L. masc. adj. *litoralis* of the shore).

Displays the following properties in addition to those given in the genus description. After 3 days on MA plates at 30 °C, colonies are creamy and approximately 2 mm in diameter. Cells are approximately 0.5–0.8 µm wide and 0.8–1.2 µm long. Grows at 10–37 °C (optimum at 30 °C) and pH 6–8 (optimum at pH 7). Growth occurs at sea salt concentrations of 1–15% (w/v) (optimum 3–5%), but no growth occurs in media containing NaCl as the only salt. Cells contain PHB granules. Gelatinase and amylase are produced. Tween 80 is not hydrolysed. Nitrate is reduced to nitrite. Positive for the following enzyme activities as tested with the API ZYM system: alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase.

Weakly positive for valine arylamidase, but negative for lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase and β-glucuronidase. Utilizes acetate, L-alanine, L-arabinose, L-arginine, D-glucose, *myo*-inositol, L-lysine, D-mannitol, peptone, L-proline, D-ribose and sucrose, but not ascorbate, DL-cysteine, dulcitol, erythritol, ethanol, glycogen, α-ketobutyric acid, lactose, L-rhamnose, oxalic acid, raffinose or succinate as sole carbon source. Cells are sensitive to (µg per disc) ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), penicillin G (10), streptomycin (10), tetracycline (30) and vancomycin (30), but resistant to nalidixic acid (30) and polymyxin B (25).

The type strain, CL-GR66^T (=KCCM 90028^T =DSM 18986^T), was isolated from coastal seawater, Korea.

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