Pelagibaca bermudensis gen. nov., sp. nov., a novel marine bacterium within the *Roseobacter* clade in the order *Rhodobacterales*

Jang-Cheon Cho[1] and Stephen J. Giovannoni[2]

[1]Division of Life and Marine Sciences, Inha University, Incheon 402-751, Republic of Korea

[2]Department of Microbiology, Oregon State University, Corvallis, OR 97331, USA

Correspondence
Jang-Cheon Cho
chojc@inha.ac.kr

A Gram-negative, chemoheterotrophic, facultatively anaerobic, slightly halophilic, oval-shaped marine bacterium, designated HTCC2601^T, was isolated from the western Sargasso Sea by high-throughput culturing involving dilution to extinction. Although the 16S rRNA gene sequence similarity between the isolate and *Salipiger mucosus* was 96·5 %, phylogenetic analyses using different treeing algorithms clearly indicated that the strain forms a distinct lineage within a clade containing the recently classified genera *Salipiger* and *Palleronia* in the order *Rhodobacterales* of the *Alphaproteobacteria*. The DNA–DNA relatedness between strain HTCC2601^T and *S. mucosus* was 26·3 %. Strain HTCC2601^T utilized a wide range of carbohydrates, including hexose monomers, sugar alcohols, organic acids and amino acids, as sole carbon sources. The DNA G+C content of strain HTCC2601^T was 65·4 mol%, and the predominant constituents of the cellular fatty acids were 18 : 1ω7*c* (79·7 %) and 11-methyl 18 : 1ω7*c* (7·5 %). The strain differed from members of the closely related genera *Salipiger* and *Palleronia* in its morphological, biochemical and ecological characteristics. On the basis of the taxonomic data obtained in this study, a novel genus and species, *Pelagibaca bermudensis* gen. nov., sp. nov., is proposed; HTCC2601^T (=KCTC 12554^T=JCM 13377^T) is the type strain of *Pelagibaca bermudensis*.

The *Roseobacter* clade (Giovannoni & Rappé, 2000) in the order *Rhodobacterales* (Garrity *et al.*, 2005) of the class *Alphaproteobacteria* is the second most abundant 16S rRNA gene-clone type in marine environments, many members of which have recently been cultivated and characterized as representing novel genera and species (Cho & Giovannoni, 2004; Martínez-Cánovas *et al.*, 2004; Martínez-Checa *et al.*, 2005; Van Trappen *et al.*, 2004; Wagner-Döbler *et al.*, 2003, 2004). Intensive cultivation approaches using a high-throughput culturing method (Connon & Giovannoni, 2002) resulted in the isolation of strain HTCC2601^T, affiliated to the *Roseobacter* clade, from the western Sargasso Sea in the Atlantic Ocean; this isolate was subjected to taxonomic investigation. Polyphasic taxonomic analyses indicated that this marine isolate represents a novel genus and species.

The original liquid culture of strain HTCC2601^T was obtained using previously described high-throughput culturing approaches (Cho & Giovannoni, 2003a). The strain was purified as single colonies on marine agar 2216 (MA; Difco) after incubation for 4 days at 25 °C, and was stored as 10 % (v/v) glycerol suspensions in liquid nitrogen. Unless otherwise indicated, bacterial cultures were grown routinely on MA at 30 °C.

Nearly complete (1460 bp) 16S rRNA gene sequences of strain HTCC2601^T were obtained as described previously (Cho & Giovannoni, 2003a) and used for phylogenetic analyses. Preliminary BLAST network searches in GenBank showed that, for strain HTCC2601^T, the closest relative with a validly published name was *Salipiger mucosus* A3^T (96·5 % sequence similarity). To clarify the phylogenetic position of HTCC2601^T, the 16S rRNA gene sequence of the strain was carefully aligned using the ARB software package (Ludwig *et al.*, 2004); only 1194 unambiguously aligned nucleotide positions were used for phylogenetic analyses in the ARB package and PAUP*, version 4.0 beta 10 (Swofford, 2002). Phylogenetic trees were generated by using neighbour joining (Saitou & Nei, 1987) with the Kimura two-parameter model (Kimura, 1980), maximum parsimony with a heuristic search (Fitch, 1971) and maximum likelihood (Felsenstein, 1981). The robustness of phylogenetic trees generated by neighbour joining and maximum parsimony was evaluated by bootstrap analyses based on 1000 resamplings. In all of the phylogenetic trees, strain HTCC2601^T and the genera *Salipiger* and *Palleronia* formed a monophyletic clade, with 62–76 % bootstrap support for a position within the order *Rhodobacterales* (Fig. 1). In spite of the high 16S

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HTCC2601^T is DQ178660.

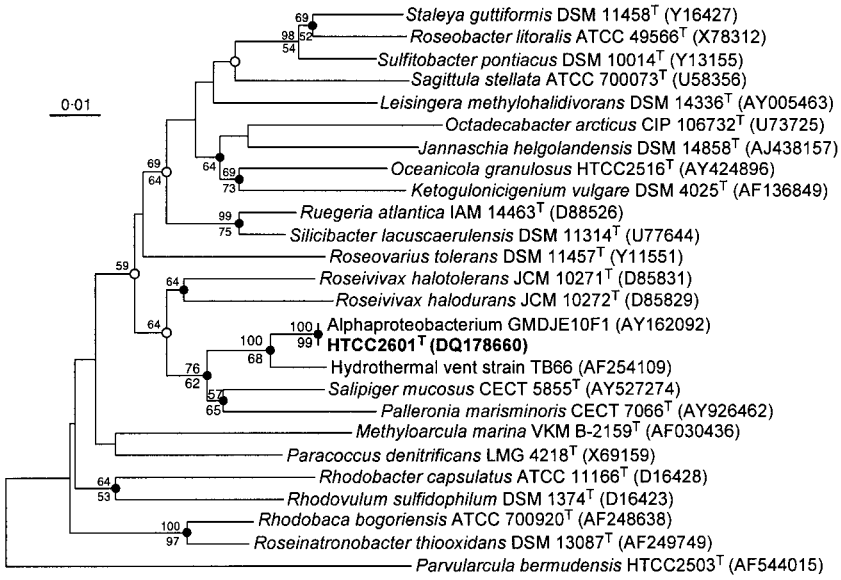

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing relationships between strain HTCC2601^T and representatives of the order *Rhodobacterales*. Bootstrap percentages (above 50 %) from both neighbour joining (above nodes) and maximum parsimony (below nodes) are shown. Filled and open circles at each node respectively indicate nodes recovered reproducibly by all treeing methods or by two treeing methods. Bar, 0·01 substitutions per nucleotide position.

rRNA gene sequence similarity (96·5 %) between strain HTCC2601^T and *S. mucosus*, the strain formed a separate subclade together with a Sargasso Sea bacterium, GMDJE10F1 (Zengler *et al.*, 2002), with 99–100 % bootstrap support within a clade containing the genera *Salipiger* and *Palleronia* (Fig. 1). The same branching-order pattern within the clade was always observed in all tree-inferring algorithms. These phylogenetic analyses suggested that strain HTCC2601^T represents a novel genus-level lineage within the *Roseobacter* clade of the order *Rhodobacterales*.

DNA–DNA hybridization was determined by dot-blot hybridization using the DIG High Prime DNA labelling and detection starter kit I (Roche Molecular Biochemicals) as described previously (Cho & Giovannoni, 2003b). Strain HTCC2601^T showed 26·3 % DNA–DNA relatedness to *S. mucosus*, the closest species with a validly published name.

The DNA G+C content of strain HTCC2601^T was analysed by using HPLC (Mesbah *et al.*, 1989) with a Platinum EPS reverse-phase C18 column. The respiratory quinones were analysed using reverse-phase HPLC (Komagata & Suzuki, 1987). Cellular fatty acid methyl esters were prepared from a culture grown on MA at 30 °C for 2 days, and analysed according to the instructions of the Microbial Identification System (MIDI). The DNA G+C content of strain HTCC2601^T was 65·4±0·4 mol%. The only respiratory quinone detected was Q-10. The major fatty acids in strain HTCC2601^T were 18 : 1ω7*c* (79·7 %), 11-methyl 18 : 1ω7*c* (7·5 %) and 16 : 0 (4·9 %). Although the predominant fatty acid component of strains HTCC2601^T, *S. mucosus* CECT 5855^T and *Palleronia marisminoris* CECT 7066^T was 18 : 1ω7*c*, these bacteria could be differentiated according to the proportions of several fatty acids, including 10 : 0 3-OH, 12 : 0 3-OH, 16 : 0 and 19 : 0 cyclo ω8*c* (Table 1).

Phenotypic characterizations were been performed as described previously (Cho & Giovannoni, 2003a), using MA as basal medium at 30 °C. Unless otherwise indicated, standard methods were employed for the phenotypic characterization of the strain, as described by Smibert & Krieg (1994). A catalase test was performed by the addition of 3·0 % H_2O_2 to fresh colonies; oxidase activity was determined using Kovács' oxidase reagent. Other biochemical tests, including the determination of nitrate reduction, acid production from glucose and indole production, were carried out on API 20NE strips (bioMérieux) according to the manufacturer's instructions. Anaerobic growth was tested on MA at 30 °C using both the Oxoid anaerobic system and the Merck Anaerocult mini C. Custom-made 48-well microplates containing 47 different carbon compounds (Cho & Giovannoni, 2003a), each at a final concentration of 0·2 % (w/v or v/v), were used to determine the utilization of various substrates as sole carbon sources; artificial sea water medium (l^{-1}: 25·0 g NaCl, 1·0 g $MgCl_2.6H_2O$, 5·0 g $MgSO_4.7H_2O$, 0·7 g KCl, 0·15 g $CaCl_2.2H_2O$, 0·5 g NH_4Cl, 0·1 g KBr, 0·27 g KH_2PO_4, 0·04 g $SrCl_2.6H_2O$, 0·025 g H_3BO_3) was used for these tests. The ability of the strain to oxidize 95 different carbon sources was determined using Biolog SF-N2 microplates (Rüger & Krambeck, 1994).

Detailed morphological, physiological and biochemical characteristics of strain HTCC2601^T are listed in the genus and species descriptions. The isolate was found to be a facultatively anaerobic, chemoheterotrophic, slightly halophilic, short rod-shaped bacterium that did not produce exopolysaccharides or poly-β-hydroxyalkanoate granules. No apparent differences in growth pattern were found between anaerobic cultures and aerobic cultures on MA in the Oxoid anaerobic system. Nitrate and nitrite were reduced on API 20NE strips. The strain utilized some simple carbohydrates, sugar alcohols, organic acids and amino acids as sole carbon sources. These phenotypic characteristics of strain HTCC2601^T clearly differentiate the strain from the most closely related species, *S. mucosus* and *Palleronia*

Table 1. Characteristics that differentiate strain HTCC2601^T from members of the phylogenetically related genera *Salipiger* and *Palleronia*

Strains: 1, HTCC2601^T; 2. *S. mucosus* CECT 5855^T (Martínez-Cánovas *et al.*, 2004); 3. *Palleronia marisminoris* CECT 7066^T (Martínez-Checa *et al.*, 2005). +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3
Source	Sea water	Hypersaline soil	Hypersaline soil
Pigment	−	−	Pink
Poly-β-hydroxyalkanoate	−	+	+
Exopolysaccharide production	−	+	+
Oxidase activity	+	+	−
Temperature range (°C)	10–40	20–40	20–37
Oxygen requirement	Facultative anaerobe	Strict aerobe	Strict aerobe
Optimum NaCl concentration (%)	3	9–10	ND*
Nitrate reduction	+	−	−
Acid production from glucose	+	−	−
Fatty acid content: (%)			
16 : 0	4·9	12·4	4·3
18 : 0	0·8	2·0	3·4
16 : 1ω7*c*	−	1·3	−
18 : 1ω7*c*	79·7	78·0	68·9
11-Methyl 18 : 1ω7*c*	7·5	1·9	−
19 : 0 cyclo ω8*c*	0·6	2·3	12·8
12 : 0 3-OH	3·4	−	−
12 : 1 3-OH	0·2	2·3	−
10 : 0 3-OH	−	−	5·0
Utilization as sole carbon sources of arabinose, glucose, fructose, cellobiose, maltose, mannose, glycerol, succinic acid, citric acid, malonic acid, propionic acid, alanine and serine	+	−	−

*The optimum Na^+ concentration for growth is 0·66 M.

marisminoris (Table 1). The genus *Salipiger* was proposed by Martínez-Cánovas *et al.* (2004) for a moderately halophlic, exopolysaccharide-producing, poly-β-hydroxyalkanoate-granule-containing, strictly aerobic bacterium that formed a distinct clade within the *Roseobacter* clade. Additionally, the genus *Palleronia* was also proposed, by the same research group (Martínez-Checa *et al.*, 2005), to be a moderately halophilic, exopolysaccharide-producing, strictly aerobic bacterium that is the phylogenetically closest neighbour with respect to the genus *Salipiger*. The important phenotypic characteristics that distinguish strain HTCC2601^T from members of the aforementioned two genera are the oxygen requirement for growth, the optimal NaCl concentration, the ability to reduce nitrate and the substrates utilized as sole carbon sources (Table 1). On the basis of these phenotypic traits, strain HTCC2601^T cannot be characterized as a member of any of the known genera within the order *Rhodobacterales*.

The polyphasic evidence, such as phenotypic data, fatty acid profiles, DNA–DNA hybridization and 16S rRNA gene phylogenetic analyses, demonstrated conclusively that strain HTCC2601^T belongs to a novel genus and species within the order *Rhodobacterales*, for which the names *Pelagibaca* gen. nov. and *Pelagibaca bermudensis* sp. nov. are proposed.

Description of *Pelagibaca* gen. nov.

Pelagibaca (Pe.la.gi.ba′ca. L. n. *pelagus* the open sea, the ocean; L. fem. n. *baca* berry, especially olive; N.L. fem. n. *Pelagibaca* an olive-shaped bacterium of the open ocean).

Cells are Gram-negative, non-motile, facultatively anaerobic, short rods (oval-shaped, 1·2–2·3 μm long, 0·6–1·1 μm wide) that multiply by binary fission. Carotenoid pigments and bacteriochlorophyll *a* are not found. Do not produce exopolysaccharides or poly-β-hydroxyalkanoate. Nitrate and nitrite are reduced. Chemoheterotrophic and slightly halophilic; require NaCl for growth. Produce acids from glucose and utilize a variety of carbon compounds as sole carbon sources. The predominant fatty acids are 18 : 1ω7*c* and 11-methyl 18 : 1ω7*c*. The only respiratory quinone detected is ubiquinone-10. The DNA G+C content is 65·4 mol%. The genus is affiliated to the *Roseobacter* clade in the order *Rhodobacterales* and currently contains only one species, the type species *Pelagibaca bermudensis*.

Description of *Pelagibaca bermudensis* sp. nov.

Pelagibaca bermudensis (ber.mu.den′sis. N.L. fem. adj. *bermudensis* from Bermuda).

In addition to having the traits reported for the genus, colonies on MA are circular, convex, cream-coloured, butyrous and 1·2–2·5 mm in diameter. Grows at 10–40 °C, optimally at 30–33 °C, but not at 4 or 44 °C. Growth occurs at pH 5·0–10·5 and 0·25–15 % NaCl, optimally at pH 8·5 and 3·0 % NaCl. Catalase- and oxidase-positive. Does not produce indole. Arginine dihydrolase-negative. Urea, aesculin and gelatin are hydrolysed. β-Glucosidase activity is present. Utilizes DL-glyceraldehyde, D-arabinose, D-glucose, D-fructose, L-rhamnose, L-sorbose, sucrose, D-trehalose, D-cellobiose, D-maltose, D-mannose, D-melibiose, D-mannitol, D-sorbitol, methanol, glycerol, pyruvic acid, succinic acid, itaconic acid, citric acid, gluconic acid, malonic acid, propionic acid, L-glutamic acid, L-ornithine, L-proline, L-lysine, L-alanine, L-serine, L-leucine and L-arginine. D-Melezitose, *myo*-inositol, L-glutamic acid, D-ribose, D-xylose, D-galactose, β-lactose, D-raffinose, adonitol, ethanol, *N*-acetyl-D-glucosamine, D-malic acid, formic acid, D-glucosamine, L-isoleucine and glycine are not utilized as sole carbon sources (tested using custom-made, 48-well plate). In tests with Biolog SF-N2 microplates, the following substrates are utilized oxidatively: α-cyclodextrin, Tweens 40 and 80, adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, α-D-glucose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, xylitol, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, citric acid, D-galacturonic acid, D-gluconic acid, γ-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, L-alanine, L-glutamic acid, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, 2,3-butanediol, glycerol, and α-DL-glycerol phosphate. Susceptible to chloramphenicol (25 μg), nalidixic acid (25 μg), carbenicillin (25 μg), tetracycline (30 μg), ampicillin (10 μg), puromycin (25 μg), erythromycin (15 μg), rifampicin (50 μg), benzylpenicillin (100 U) and gentamicin (10 μg), but resistant to kanamycin (30 μg), vancomycin (30 μg) and cycloheximide (50 μg). The cellular fatty acids are composed of 18 : 1ω7*c* (79·7 %), 11-methyl 18 : 1ω7*c* (7·5 %), 16 : 0 (4·9 %), 12 : 0 3-OH (3·4 %), 18 : 0 (0·8 %), 19 : 0 cyclo ω8*c* (0·6 %), 18 : 1ω9*c* (0·6 %), 10 : 0 3-OH (0·4 %), 12 : 1 3-OH (0·2 %) and 17 : 0 (0·2 %).

The type strain, HTCC2601^T (=KCTC 12554^T=JCM 13377^T), was isolated from the Bermuda Atlantic Time Series Station in the western Sargasso Sea, Atlantic Ocean.

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