Porphyrobacter donghaensis sp. nov., isolated from sea water of the East Sea in Korea

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Two Gram-negative, motile, non-spore-forming, bacteriochlorophyll *a*-containing slightly halophilic strains, SW-132^T and SW-158, were isolated from sea water of the East Sea in Korea, and subjected to a polyphasic taxonomic study. The two isolates were characterized chemotaxonomically as having Q-10 as the predominant respiratory lipoquinone and major amounts of unsaturated fatty acids C_{18:1}ω7c and C_{17:1}ω6c. The DNA G+C contents of the two strains were in the range 66.8–65.9 mol%. The 16S rRNA gene sequences of strains SW-132^T and SW-158 were 99.9% (1 nt difference) similar and their mean level of DNA–DNA relatedness was 86%. The 16S rRNA gene sequence analysis showed that strains SW-132^T and SW-158 are phylogenetically closely related to *Porphyrobacter* species and *Erythromicrobium ramosum*. Similarities between the 16S rRNA gene sequences of strains SW-132^T and SW-158 and the type strains of *Porphyrobacter* species and *E. ramosum* ranged from 97.8 to 99.0%. DNA–DNA relatedness data indicated that strains SW-132^T and SW-158 are members of a genomic species that is separate from the four *Porphyrobacter* species. On the basis of phenotypic and phylogenetic data and genetic distinctiveness, strains SW-132^T (= KCTC 12229^T = DSM 16220^T) and SW-158 (= KCTC 12230) are classified as a novel *Porphyrobacter* species, for which the name *Porphyrobacter donghaensis* sp. nov. is proposed.

The genus *Porphyrobacter* was proposed by Fuerst *et al.* (1993) with a single species, *Porphyrobacter neustonensis*. Phylogeny based on 16S rRNA gene sequences has shown that the genus *Porphyrobacter* belongs to the α -subclass of the *Proteobacteria* (Anzai *et al.*, 2000; Hiraishi *et al.*, 2002; Rainey *et al.*, 2003). *Porphyrobacter* species are red or orange in colour and synthesize bacteriochlorophyll *a* (Fuerst *et al.*, 1993; Hanada *et al.*, 1997; Hiraishi *et al.*, 2002; Rainey *et al.*, 2003). At present, the genus *Porphyrobacter* comprises four species with validly published names: *P. neustonensis* (Fuerst *et al.*, 1993), *Porphyrobacter tepidarius* (Hanada *et al.*, 1997), *Porphyrobacter sanguineus* (Hiraishi *et al.*, 2002) and *Porphyrobacter cryptus* (Rainey *et al.*, 2003). In this study, we report on the detailed taxonomic characterization of two slightly halophilic, reddish-orange-pigmented, *Porphyrobacter*-like bacterial strains, SW-132^T and SW-158, which were isolated from sea water of the East Sea in Korea.

Strains SW-132^T and SW-158 were isolated by the usual dilution plating technique on marine agar 2216 (MA; Difco)

at 30 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. The presence of flagella was investigated using transmission electron microscopy using cells from exponentially growing cultures. The cells were negatively stained with 1% (w/v) phosphotungstic acid and the grids were examined after air-drying with a Philips CM-20 transmission electron microscope. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA and MA supplemented with nitrate that had been prepared anaerobically using nitrogen. Growth in the absence of NaCl was investigated in trypticase soy broth without NaCl. Growth at various NaCl concentrations was investigated in marine broth (Difco) or trypticase soy broth (Difco). Growth at various temperatures (4–50 °C) was measured on MA. Catalase and oxidase activities and hydrolysis of casein, starch, Tween 20, Tween 40, Tween 60 and Tween 80 were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA with the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and nitrate reduction were studied as described previously (Lanyi, 1987) with the modification that artificial sea water was used for the preparation of media. The artificial sea water contained (per litre of distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SW-132^T and SW-158 are AY559428 and AY559429, respectively.

H₂S production was tested for as described previously (Bruns *et al.*, 2001). For *in vivo* pigment-absorption spectrum analysis, two strains were cultivated aerobically in the dark at 30 °C in *Erythromicrobium/Roseococcus* medium (Yurkov *et al.*, 1994; DSMZ medium no. 767) with the modification that glucose was used instead of acetate. The cultures were washed twice by centrifugation using a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by sonication with a Branson sonifier 450. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer. Susceptibility to antibiotics was detected on agar plates by using antibiotic discs containing chloramphenicol (100 µg), penicillin G (20 U), polymyxin B (100 U) or streptomycin (50 µg). Utilization of substrates as sole carbon and energy sources was tested according to the method of Baumann & Baumann (1981), using supplementation with 2% (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1% (v/v) vitamin solution (Staley, 1968).

Cell biomass of strains SW-132^T and SW-158 for respiratory lipoquinone analysis and for DNA extraction was produced in marine broth at 30 °C. Respiratory lipoquinones were analysed as described previously (Komagata & Suzuki, 1987), using reversed-phase HPLC. Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996) with the exception that ribonuclease T1 was used together with ribonuclease A. For fatty acid methyl ester analysis, cell mass of strains SW-132^T and SW-158 was obtained from agar plates after cultivation for 7 days on MA at 30 °C. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined by using the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The 16S rRNA gene was amplified by using a PCR with two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989) with photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the remaining three values were used to calculate similarity values. The DNA relatedness values quoted are the means of the three values.

Strains SW-132^T and SW-158 were similar in terms of most morphological, cultural, physiological and biochemical characteristics. The two strains grew optimally at 30–37 °C and in the presence of 2% (w/v) NaCl, and they grew without NaCl. Strain SW-132^T did not grow in the presence

of >6% NaCl, while strain SW-158 did not grow in the presence of >7% NaCl. Sucrose and acetate were utilized by strain SW-132^T, but not by strain SW-158. Both strains produced bacteriochlorophyll *a* aerobically in the dark. The sonicated cell extracts of the two strains showed absorption maxima at approximately 467, 590, 808 and 867 nm, which indicated the presence of carotenoids and bacteriochlorophyll *a* (Fig. 1). Acetone/methanol extracts had *in vitro* maximal absorption peaks at 766–768 nm, confirming the presence of bacteriochlorophyll *a* (data not shown). Similar *in vivo* absorption spectra were produced for the other three *Porphyrobacter* species, with the exception of *P. sanguineus*, which shows a minor difference in absorption spectrum (Fuerst *et al.*, 1993; Hanada *et al.*, 1997; Hiraishi *et al.*, 2002; Rainey *et al.*, 2003). Other morphological, cultural, physiological and biochemical characteristics are shown in Table 1 or are given in the species description (see below). Phenotypic characteristics differentiating strains SW-132^T and SW-158 from *Porphyrobacter* species are summarized in Table 1.

The predominant respiratory lipoquinone detected in strains SW-132^T and SW-158 was Q-10, at peak area ratios of approximately 78–86%; Q-9 and Q-8 were present as minor components. Strains SW-132^T and SW-158 showed cellular fatty acid profiles that consisted of straight-chain, unsaturated and hydroxy fatty acids; the major fatty acids were C_{18:1}ω7c and C_{17:1}ω6c (Table 2). Although similar fatty acid profiles were observed in other *Porphyrobacter* species, there were differences in the proportions of major fatty acids, which might be caused by different cultivation conditions, e.g. temperature and media used for cultivation (Hiraishi *et al.*, 2002; Rainey *et al.*, 2003). The amounts of unsaturated fatty acid C_{18:1}ω7c were lower than those of other *Porphyrobacter* species, whereas the fatty acid C_{17:1}ω6c was a minor component in *P. neustonensis* and *P. tepidarius* (Hiraishi *et al.*, 2002; Rainey *et al.*, 2003). The DNA G+C contents of strains SW-132^T and SW-158 were

Fig. 1. *In vivo* absorption spectra of sonicated cell extracts of strains SW-132^T and SW-158.

Table 1. Differential phenotypic characteristics of *Porphyrobacter* species

Taxa: 1, *P. neustonensis*, data from Fuerst *et al.* (1993); 2, *P. tepidarius*, data from Hanada *et al.* (1997); 3, *P. sanguineus*, data from Hiraishi *et al.* (2002); 4, *P. cryptus*, data from Rainey *et al.* (2003); 5, *P. donghaensis*, data from this study. *n*, Number of strains; +, positive reaction; –, negative reaction; ND, not determined; v, variable reaction. Data for the type strain are in parentheses. All species are aerobic, Gram-negative, non-sporulating, positive for the presence of bacteriochlorophyll *a*, positive for the utilization of D-glucose and negative for the utilization of citrate.

Characteristic	1 (<i>n</i> =4)	2 (<i>n</i> =1)	3 (<i>n</i> =2)	4 (<i>n</i> =2)	5 (<i>n</i> =2)
Cell morphology	Pleomorphic	Ovals or short rods	Pleomorphic	Short rods	Pleomorphic
Catalase	+	ND	+	+	+
Oxidase	–	ND	+	+	+
Motility	+	–	+	+	–
Growth at 0% NaCl	+	+	–	ND	+
Hydrolysis of:					
Aesculin	V(+)	ND	ND	+	+
Casein	V(–)	ND	–	ND	–
Gelatin	–	–	–	+	–
Starch	–	+	–	+	+
Tween 80	+	+	–	ND	+
Utilization of:					
D-Fructose	V(–)	–	–	–	–
D-Galactose	+	ND	ND	–	–
D-Cellobiose	V(–)	ND	+	+	+
D-Mannose	+	ND	–	–	–
D-Trehalose	V(+)	ND	ND	–	–
D-Xylose	+	ND	ND	+	v(+)
L-Arabinose	–	ND	–	–	–
Sucrose	+	ND	+	+	V(–)
Acetate	–	+	+	–	V(–)
Succinate	V(+)	–	–	–	+
L-Malate	–	–	–	–	+
Pyruvate	V(+)	–	+	+	+
Lactose	–	ND	–	–	–
L-Glutamate	ND	+	+	+	–
Max. temperature (°C)	37	52	37	<60	<50
Optimum growth temperature (°C)	30	40–48	30	50	30–37
DNA G+C content (mol%)	65.7–66.4	65.0	63.8–64.0	66.2	65.9–66.8

66.8 and 65.9 mol%, respectively. The almost-complete 16S rRNA gene sequences of strains SW-132^T and SW-158, comprising 1440 nt (approx. 96% of the *Escherichia coli* 16S rRNA gene sequence), were determined in this study. There was a 1 nt difference between the 16S rRNA gene

sequence of strain SW-132^T and that of strain SW-158. The 16S rRNA gene sequence analysis revealed that the two strains have the highest levels of 16S rRNA gene similarity to members of the *α-Proteobacteria*, particularly to the genus *Porphyrobacter* and to *Erythromicrobium ramosum*.

Table 2. Cellular fatty acid profiles of strains SW-132^T and SW-158

Fatty acids representing <1.0% in two strains were omitted. i, iso; ND, not detected.

Strain	Fatty acid composition (%)*												
	C _{14:0} 2-OH	C _{15:0}	C _{16:0}	C _{15:0} 2-OH	i-C _{16:0} 3-OH	C _{16:0} 2-OH	C _{17:1ω6c}	C _{17:1ω8c}	i-C _{17:0}	C _{17:0}	C _{18:1ω5c}	C _{18:1ω7c}	
SW-132 ^T	6.8	1.3	4.1	5.5	1.4	1.5	34.7	2.7	ND	1.0	3.4	35.8	
SW-158	8.2	1.4	5.4	9.4	1.2	ND	31.5	2.7	2.4	1.6	1.3	34.2	

Phylogenetic trees based on 16S rRNA gene sequences showed that strains SW-132^T and SW-158 fall within the radiation of the cluster comprising *Porphyrobacter* species and *Erythromicrobium ramosum* (Fig. 2). Strains SW-132^T and SW-158 exhibited levels of 16S rRNA gene sequence similarity to type strains of *Porphyrobacter* species and *Erythromicrobium ramosum* that were between 97.8 and 99.0%. The levels of 16S rRNA gene sequence similarity between strains SW-132^T and SW-158 and other species included in the phylogenetic analysis ranged from 97.9% (*Erythrobacter litoralis* DSM 8509^T) to 91.7% (*Sphingobium yanoikuyae* IFO 15102^T). Strains SW-132^T and SW-158 exhibited a mean level of DNA–DNA relatedness of approximately 86% when their DNAs were used individually as labelled DNA probes for cross-hybridization. Levels of DNA–DNA relatedness between two strains and the type strains of *Porphyrobacter* species were in the range 9.4–20.3%.

Phylogenetic analysis based on 16S rRNA gene sequences placed strains SW-132^T and SW-158 within a phyletic cluster comprising *Porphyrobacter* species and *Erythromicrobium ramosum* (Fig. 2). The relationship between this cluster and the clade that comprised *Erythrobacter* species was supported by a bootstrap confidence level of 100% (Fig. 2). The genera *Porphyrobacter* and *Erythromicrobium* could not be clearly distinguished since they are not well defined chemotaxonomically and are intermixed phylogenetically (Rainey *et al.*, 2003). The possibility that the genera *Porphyrobacter* and *Erythromicrobium* have to be combined into a single genus was proposed by Rainey *et al.* (2003). This reclassification may be necessary to aid the exact classification of species or strains that are related to the three genera. However, there is still no such proposal, or it may not be appropriate at this time, which leads us to place the two isolates into one of the three genera. Accordingly, since strains SW-132^T and SW-158 exhibit the highest levels of 16S rRNA gene sequence similarity to some *Porphyrobacter* species, it is better to place them in the genus *Porphyrobacter* before taxonomic criteria regarding the three genera are proposed.

Strains SW-132^T and SW-158 show very similar phenotypic characteristics. The two isolates are also phylogenetically and genetically similar. In view of these combined data, strains SW-132^T and SW-158 should be considered as members of the same species (Wayne *et al.*, 1987). There are some phenotypic properties differentiating strains SW-132^T and SW-158 from *Porphyrobacter* species, including the ability to utilize some substrates (Table 1). The level of DNA–DNA relatedness, together with differential phenotypic properties and 16S rRNA gene sequence similarity data, justify the taxonomic discrimination of SW-132^T and SW-158 from all *Porphyrobacter* species with validly published names (Wayne *et al.*, 1987). Therefore, on the basis of the data presented, strains SW-132^T and SW-158 should be classified in the genus *Porphyrobacter* as members of a novel species, for which the name *Porphyrobacter donghaensis* sp. nov. is proposed.

Description of *Porphyrobacter donghaensis* sp. nov.

Porphyrobacter donghaensis (dong.ha.en'sis. N.L. masc. adj. *donghaensis* of Donghae, the Korean name for the East Sea in Korea from which the strains were isolated).

Cells are pleomorphic: cocci, ovals or rods (0.6–0.8 × 0.9–2.5 µm) are present. Colonies on MA are smooth, circular, convex, reddish-orange in colour and 0.5–0.8 mm in diameter after 7 days incubation at 30 °C. Growth occurs at 10 and 45 °C but not at 4 or 50 °C. The optimal pH for growth is pH 7.0–8.0; growth is observed at pH 5.0 but not at pH 4.5. Optimal growth occurs in the presence of 2% (w/v) NaCl; growth does not occur in the presence of >7% NaCl. Growth does not occur under anaerobic conditions on MA and on MA with nitrate. Bacteriochlorophyll *a* and carotenoids are present. Urease-negative. Tween 20, Tween 40, Tween 60 and tyrosine are hydrolysed. Hypoxanthine and xanthine are not hydrolysed. H₂S is not produced. Sensitive to chloramphenicol (100 µg) and penicillin G (20 U). Resistant to polymyxin B (100 U) and streptomycin (50 µg). Maltose is utilized. Benzoate and

Fig. 2. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic positions of strains SW-132^T and SW-158 and representatives of some related taxa. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50% or greater. Bar, 0.01 substitution per nucleotide position. *Rhodospirillum rubrum* ATCC 11170^T was used as the outgroup.

formate are not utilized. The predominant respiratory lipoquinone is Q-10. The major fatty acids are C_{18:1}ω7c and C_{17:1}ω6c. The DNA G + C content is 65.9–66.8 mol% (66.8 mol% for the type strain) (determined by HPLC). Other phenotypic characteristics are given in Tables 1 and 2.

The type strain, SW-132^T (=KCTC 12229^T=DSM 16220^T), and reference strain SW-158 (=KCTC 12230) were isolated from sea water from the East Sea in Korea.

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