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Short Communication

Porphyrobacter colymbi sp. nov. isolated from swimming pool water in Tokyo, Japan

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The genus *Porphyrobacter* was proposed by Fuerst et al. (1993) and, at the time of this writing, comprises six recognized species: *P*. *neustonensis* (Fuerst et al., 1993), *P*. *tepidarius* (Hanada et al., 1997), *P*. *sanguineus* (Hiraishi et al., 2002), *P*. *cryptus* (Rainey et al., 2003), *P*. *donghaensis* (Yoon et al., 2004) and *P*. *dokdonensis* (Yoon et al., 2006). Colonies of *Porphyrobacter* species are red or orange in color and synthesize bacteriochlorophyll *a* (Fuerst et al., 1993; Hanada et al., 1997; Hiraishi et al., 2002; Rainey et al., 2003; Yoon et al., 2004, 2006). These strains have been isolated from various environments of fresh water (Fuerst et al., 1993), hot spring water (Hanada et al., 1997; Rainey et al., 2003) and sea water (Hiraishi et al., 2002; Yoon et al., 2004, 2006). In this study, we describe the morphological, physiological and genetic characteristics of the new isolate TPW-24^T isolated from swimming pool water and propose that this strain represents a new species in the genus *Porphyrobacter*, *Porphyrobacter colymbi*.

Strain TPW-24^T was isolated from swimming pool water (free residual chlorine: 0.4 mg/L) from Metropolitan Tokyo, Japan in August of 2009 using a plating method with standard agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C for 24 h. The isolated strain was preserved in the Microbank (Iwaki & Co., Ltd., Tokyo, Japan) and stored at −80°C.

The almost complete sequence of the 16S rRNA gene was determined using the MicroSeq 16S rRNA gene bacterial identification kit (Applied Biosystems). A multiple sequence alignment analysis was performed using the CLUSTAL W software program (Thompson et al., 1994) and gaps and unidentified base positions were deleted using the BioEdit (Hall, 1999) software package. Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980) without alignment gaps and unidentified base positions were taken into account during distance calculations. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) and the bootstrap values were calculated on the basis of 1,000 replications (Felsenstein, 1985).

The quantitative microplate DNA-DNA hybridization test on the TPW-24^T strain and *P*. *donghaensis* KCTC 12229^T was performed at the TechnoSuruga Laboratory in Shizuoka Prefecture, Japan, as previously described (Ezaki et al., 1989). The methods used to isolate and purify DNA have been previously described (Hamamoto and Nakase, 1995). The levels of DNA re-

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latedness were determined using the immobilized microplate method performed at 51°C in the presence of 50% formamide. A fluorescence plate reader (Genios, Tecan Japan Co., Ltd., Kanagawa, Japan) was used to measure the intensity of fluorescence. Examinations were performed in triplicate and the mean values were calculated.

The Gram reaction was determined using the Nissui Gram stain kit (Nissui Pharmaceutical Co., Ltd., Tokyo) according to the manufacturer's instructions. The shape and motility of bacterial cells were observed under a phase-contrast microscope (1,000×) with cell suspensions made from cultures grown on R2A agar (Nihon Pharmaceutical Co., Ltd., Tokyo) at 30°C for 3 days. Scanning electron microscope observations were performed on samples fixed to glass filters at the Osaka Prefectural Institute of Public Health, Japan. Samples were fixed with 2% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), critical-point-dried, sputter-coated with osmium and visualized using a scanning electron microscope (JSM-T100, JEOL Ltd., Tokyo, Japan). Growth at various temperatures (4 and 15–45°C using increments of 5°C) was determined on R2A broth (100 ml). Growth at various NaCl concentrations (0.1, 0.3, 0.5 and 1.0–5.0% using increments of 1.0%) was investigated in R2A broth. The pH range for growth was determined in R2A broth that had been adjusted to various pH values (pH 4.0–10.0 using increments of 1.0 pH units) prior to sterilization by the addition of HCl or NaOH. Growth at various NaCl concentrations and pH values was tested at 30°C. The turbidity (OD 660 nm) of each medium was measured after incubation for 2, 5 and 7 days. Based on the rise in turbidity, we judged the results to be positive. The catalase activities were examined using 3% H_2O_2 and the oxidase activities were examined using a test paper containing tetramethyl-phenylenediamine dihydrochloride (Nissui Pharmaceutical Co., Ltd., Tokyo).

Biochemical tests were performed using API 20NE, API 50CH and API ZYM identification systems (SYSMEX bioMérieux Co., Ltd., Tokyo). API ZYM strips were read after 48 h of incubation at 30°C. The API 20NE and API 50CH were read after 3 days of incubation at 30°C. AUX medium was used as the API 50CH test basal medium, and positive reactions were judged based on the turbidity of the medium after incubation.

Antibiotic susceptibility was determined using Etest (SYSMEX bioMérieux Co., Ltd., Tokyo) according to the attached technical guide. The drugs tested were ampicillin (ABPC), piperacillin (PIPC), cefotaxime (CTX), cefuroxime (CXM), ceftazidime (CAZ), amikacin (AMK), gentamicin (GM), azithromycin (AZM), erythromycin (EM), minocycline (MINO), tetracycline (TC), chloramphenicol (CP), vancomycin (VCM), imipenem (IPM), meropenem (MEPM), ciprofloxacin (CPFX), ofloxacin (OFLX), sparfloxacin (SPFX), fosfomycin (FOM) and sulfamethoxazole-trimethoprim (ST) (total 20 drugs). Bacterial cell suspensions (0.3 ml) were dripped onto 60 ml of R2A agar in a 150-mm dish (Corning, Inc., USA) and smeared over the surface using a Conradi stick, after which Etest strips were securely attached to the medium. The plates were cultured at 30°C for 3 days and the growth inhibition zone formed around the strip was read. MIC was judged by macroscopically reading the graduation at which the end of the growth inhibition zone and the strip crossed.

Quinone analyses, cellular fatty acid composition and the guanine-plus-cytosine (G+C) content were performed by the TechnoSuruga Laboratory (Shizuoka, Japan). Strain TPW-24^T was cultured at 30°C for 3 days in R2A agar medium for these analyses. Quinones were extracted from freeze-dried cells and analyzed using the HPLC system (Waters 600 series, Nihon Waters K.K., Tokyo) (Nishijima et al., 1997). The analysis of cellular fatty acid composition was performed using a Sherlock Microbial Identification System (Version 4.5) (MIDI, Newark, DE). The G+C content of DNA from the isolate was determined using the method proposed by Katayama et al. (1984). Genomic DNA was extracted from cultured cells using the phenol extraction method and nucleotides were obtained from DNA using hydrolysis of nuclease P1. The various peaks of nucleotide were isolated and detected using HPLC (LC-10, Shimadzu Co., Kyoto) and the G+C content was calculated.

For in vivo pigment-absorption spectrum analysis, the strain was cultivated aerobically at 30°C for 3 days in R2A agar medium. The cultures were washed twice by centrifugation using phosphate buffer and disrupted by sonication for 10 min with a Branson sonifier 250. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Hitachi Ratio Beam Spectrophotometer U-1800 (Hitachi High-Technologies Co., Tokyo).

The almost complete 16S rRNA gene sequence (1,445 bp) of strain TPW-24^T was determined (accession number: AB702992). A neighbor-joining tree

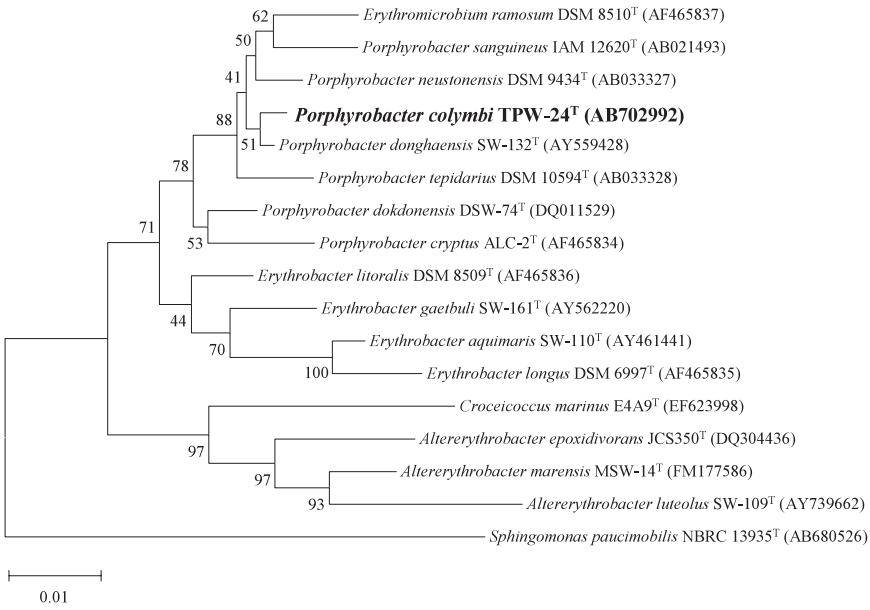

Fig. 1. A phylogenetic tree constructed using the neighbor-joining method based on 16S rRNA partial region sequences (1,373 bp) of strain TPW-24^T (accession number: AB702992) and related bacteria.

Sphingomonas paucimobilis NBRC 13935^T (AB680526) served as an out group. The data set was resampled 1,000 times using the bootstrap option and the percentage values are given at the nodes. The scale bar indicates the number of substitutions per nucleotide position.

showed that strain TPW-24^T was closely related to the type strain of the six recognized species of the genus *Porphyrobacter* (Fig. 1). Strain TPW-24^T formed a distinct cluster with *P*. *donghaensis* SW-132^T in the phylogenetic tree: however, the significant bootstrap was very low (51%). The values of 16S rRNA gene sequence similarity between strain TPW-24^T and the closest relatives were 99.6% (*P*. *donghaensis* SW-132^T =KCTC 12229^T and SW-158 =KCTC 12230) (Yoon et al., 2004) and 99.3% (*P*. *tepidarius* OT-3^T =DSM 10595) (Hanada et al., 1997). The DNA-DNA hybridization similarity values were obtained under optimal conditions, namely 51°C. The TPW-24^T isolate showed less than 8.0% similarity to the strain of *P*. *donghaensis* SW-132^T (=KCTC 12229^T), which had the closest relationship in the phylogenetic tree based on the 16S rRNA of the genus *Porphyrobacter* (Fig. 1). This result clearly demonstrated that the TPW-24^T strain should be categorized as a new taxon (Wayne et al., 1987).

In Table 1, we list the important phenotypic characteristics of strain TPW-24^T and compare them with those of the most closely related strain *P*. *donghaensis* KCTC 12229^T. The cells of strain TPW-24^T were Gram-negative, motile, non-spore-forming rods. A photomicrograph of strain TPW-24^T cultured on R2A agar at 30°C for 3 days is shown in Fig. 2. The size of these cells measured 0.3−0.4×1.0−1.3 μm. Colonies of strain TPW-24^T were orange-pigmented with a diameter of approximately 1.0 mm on R2A agar after 3 days of incubation at 30°C. Growth occurred at 20−40°C and pH 7.0−9.0, with optimal growth occurring at 35°C and pH 9.0 (Fig. 3). Growth did not occur in the presence of >1.0% NaCl (Fig. 3). Both oxidase and catalase reaction were positive.

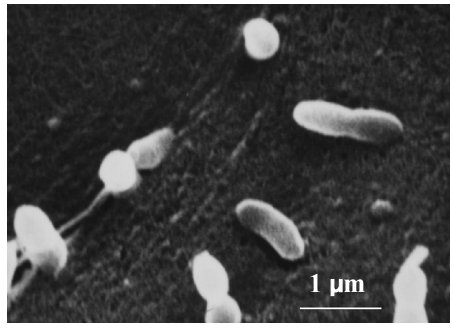

Fig. 2. A photomicrograph of the cells of strain TPW-24^T cultured on R2A agar at 30°C for 3 days.

The scale bar indicates 1 μm.

The API 20NE identification system gave positive re-

Table 1. Comparative phenotypic characteristics of *P. colymbi* TPW-24^T and *P. donghaensis* KCTC 12229^T.

Characteristics	*P. colymbi* TPW-24^T	*P. donghaensis* KCTC 12229^T
Gram staining	−	−
Cell morphology	Rods	Pleomorphic
Motility	+	+
Optimum growth temperature (°C)	35	25−35
Optimum growth pH	9	7−8
NaCl (%) range for growth	≦0.5	≦3
Growth in R2A broth	+	−
Catalase	+	+
Oxidase	+	+
Reduction of nitrate	−	−
Hydrolysis of:		
Esculin	+	+
Gelatin	+	−
Starch	+	+
Utilization of:		
D-Glucose	+	+
L-Arabinose	+	−
D-Galactose	−	−
D-Fructose	−	−
D-Cellobiose	+	+
D-Mannose	+	−
D-Trehalose	−	−
D-Xylose	+	+
D-Sucrose	+	+
Enzyme actibity (API ZYM)		
Alkaline phosphatase	+	+
Acid phosphatase	+	−
Naphthol-AS-BI-phosphohydrolase	+	−
Susceptibility (MIC, μg/ml)		
Erythromycin	4	2
Tetracycline	0.25	0.125
Ofloxacin	4	2
Ciprofloxacin	2	0.25
Sulfamethoxazole-trimethoprim	0.016	> 32
Isolation source	Pool water	Sea water[a]
Respiratory lipoquinone	Q-10	Q-10[a]
Cellular fatty acid	18:1ω7c	18:1ω7c, 17:1ω6c[a]
DNA G+C content (mol%)	66.9	66.8[a]

[a] Data from Yoon et al. (2004).

sults for esculin, gelatin, *p*-nitrophenyl-β-D-galactopyranoside, D-glucose, L-arabinose, D-mannose and maltose (API code number 0473204). API 50CH strips used as the sole carbon source showed positive results for L-arabinose, D-xylose, D-glucose, D-mannose, esculin, D-cellobiose, D-maltose, D-sucrose and starch. The differential phenotypic characteristics of strain TPW-24^T and *P. donghaensis* KCTC 12229 are summarized in Table 1.

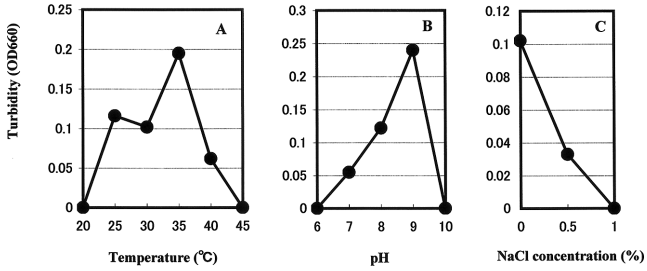

Fig. 3. Growth of strain TPW-24^T under various conditions in R2A broth for 2 days.
B and C at 35°C.

Strain TPW-24^T was positive for alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase (Table 1). The results were negative for esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, ß-galactosidase, ß-glucuronidase, α-glucosidase, ß-glucosidase, *N*-acetyl-ß-glucosaminidase, α-mannosidase and α-fucosidase. Although *P. donghaensis* KCTC 12229^T showed negative results for acid phosphatase and naphthol-AS-BI-phosphohydrolase, the enzyme activities were very useful for identifying these species.

The major cellular fatty acids of strain TPW-24^T were $C_{18:1}\omega7c$ (67.0%), $C_{14:0}2OH$ (8.2%) and $C_{17:1}\omega6c$ (5.9%). This fatty acid profile was similar to that of *P. neustonensis* (Fuerst et al., 1993), *P. sanguineus* (Hiraishi et al., 2002), *P. cryptus* (Rainey et al., 2003) and *P. donghaensis* (Yoon et al., 2004); however, it differed from that of *P. dokdonensis* (Yoon et al., 2006). As with other members of the genus *Porphyrobacter* (Yoon et al., 2004, 2006), the predominant quinone of strain TPW-24^T was ubiquinone-10 (Q-10). The DNA G+C content of strain TPW-24^T was 66.9 mol%, and the value was similar to that of *P. cryptus* (Rainey et al., 2003) and *P. donghaensis* (Yoon et al., 2004).

Strain TPW-24^T was susceptible to MINO (MIC< 0.016 μg/ml), ST (MIC=0.016 μg/ml), CP, IPM, MEPM and VCM (MIC=0.125 μg/ml), TC and AZM (MIC= 0.25 μg/ml), CXM and CPFX (MIC=2 μg/ml), ABPC, GM, EM OFLX and SPFX (MIC=4 μg/ml) and CAZ and AMK (MIC=8 μg/ml) and showed resistance to CTX (MIC>32 μg/ml), PIPC (MIC>256 μg/ml) and FOM

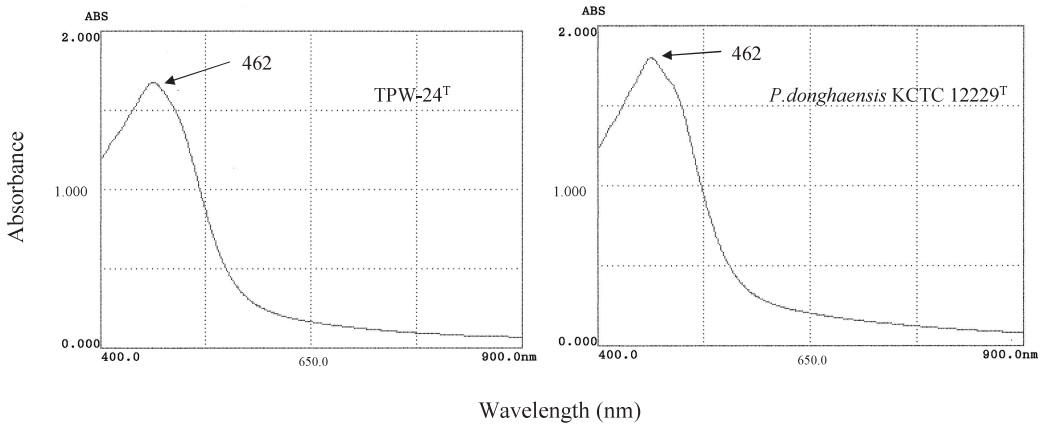

Fig. 4. In vivo absorption spectra of sonicated cell extracts of both strains TPW-24^T and *P. donghaensis* KCTC 12229^T.

(MIC>1,024 μg/ml). However, as shown in Table 1, the susceptibility of strain TPW-24^T differed from that of *P. donghaensis* KCTC 12229^T.

The sonicated cell extracts of strain TPW-24^T and *P. donghaensis* KCTC 12229^T showed absorption maxima at approximately 462 nm, which indicated the presence of carotenoids (Fig. 4). However, the two strains did not produce bacteriochlorophyll *a*. Due to differences in both medium and culture conditions, it was thought that different results had been obtained (Fuerst et al., 1993; Hanada et al., 1997; Hiraishi et al., 2002; Rainey et al., 2003; Yoon et al., 2004).

We examined the properties of strain TPW-24^T isolated from swimming pool water in Tokyo, Japan. Some characteristics of this strain are shown in Table 1. According to the comparison of 16S rRNA gene sequences (1,445 bp) and phylogenetic analysis data (Fig. 1), strain TPW-24^T was classified as a member of the genus *Porphyrobacter*. Strain TPW-24^T formed a distinct cluster with *P. donghaensis* SW-132^T (=KCTC 12229^T) in the phylogenetic tree: however, the significant bootstrap was very low (51%). The value of 16S rRNA gene sequence similarity between strain TPW-24^T and *P. donghaensis* SW-132^T (=KCTC 12229^T) was 99.6%. The phylogenetic analyses based on 16S rRNA gene sequences demonstrated that the two organisms are highly genetically related and belong to a single genus. However, the DNA-DNA hybridization level between the two strains (8.0%) was lower than 70%, the lower limit of the value indicative of single species status (Wayne et al., 1987). Therefore, it is concluded that TPW-24^T and *P. donghaensis* SW-132^T (=KCTC 12229^T) may be classified as different species in a single genus. It is evident from the phylogenetic data that strain TPW-24^T represents a novel species of the genus *Porphyrobacter*. Moreover, the two strains exhibit some phenotypic differences that are useful as diagnostic features for species differentiation, e.g. NaCl tolerance, gelatin hydrolysis, carbon nutrition and enzyme activity (Table 1).

Description of *Porphyrobacter colymbi* sp. nov.

Porphyrobacter colymbi (co'lym. bi. L. gen. n. *colymbi*, of a swimming pool, thus indicating the site of isolation of the type strain).

The cells are Gram-negative, non-spore-forming rods, measuring 0.3−0.4×1.0−1.3 μm. The cells are motile. Colonies are smooth and orange-pigmented and approximately 1.0 mm in diameter on R2A agar after 3 days at 30°C. Growth occurs at 20−40°C and pH 7.0−9.0 but not at 4 or 45°C and pH 6.0 or 10.0. Optimum growth conditions are 35°C and pH 9.0. Does not grow in the presence of >1.0% NaCl. Oxidase- and catalase-positive. Esculin, gelatin and starch are hydrolysed. Hydrogen sulfide and indole are not produced. Nitrate is not reduced. Utilization of D-glucose, L-arabinose, D-cellobiose, D-mannose, D-xylose and D-sucrose are all positive. Alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present. Sensitive to MINO (MIC<0.016 μg/ml) and ST (MIC=0.016 μg/ml). Resistant to CTX (MIC>32 μg/ml), PIPC (MIC>256 μg/ml) and FOM (MIC>1,024 μg/ml). The major quinone is ubiquinone-10 (Q-10). The major cellular fatty acids are $C_{18:1}$ ω7c, $C_{14:0}$ 2OH and $C_{17:1}$ ω6c. The G+C content of the type strain is 66.9 mol%. The type strain TPW-24^T (=JCM 18338^T =KCTC 32078^T) was isolated from swimming pool water in Tokyo, Japan.

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References

Ezaki, T., Hashimoto, Y., and Yabuuchi, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int*. *J*. *Syst*. *Bacterial*., **39**, 224–229.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, **39**, 783–791.

Fuerst, J. A., Hawkins, J. A., Holmes, A., Sly, L. I., Moore, C. J., and Stackebrandt, E. (1993) *Porphyrobacter neustonensis* gen. nov., sp. nov., an aerobic bacteriochlorophyll-synthesizing budding bacterium from fresh water. *Int*. *J*. *Syst*. *Bacterial*., **43**, 125–134.

Hall, T. A. (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucleic Acids Symp*. *Ser*., **41**, 95–98.

Hamamoto, M. and Nakase, T. (1995) Ballistosporous yeasts found on the surface of plant materials collected in New Zealand. *Antonie van Leewenhoek*, **67**, 151–171.

Hanada, S., Kawase, Y., Hiraishi, A., Takaichi, S., Matsuura, K., Shimada, K., and Nagashima, K. V. P. (1997) *Porphyrobacter tepidarius* sp. nov., a moderately thermophilic aerobic photosynthetic bacterium isolated from a hot spring. *Int*. *J*. *Syst*. *Bacteriol*., **47**, 408–413.

Hiraishi, A., Yonemitsu, Y., Matsushita, M., Shin, Y., K., Kuraishi, H., and Kawahara, K. (2002) Characterization of *Porphyrobacter sanguineus* sp. nov., an aerobic bacteriochlorophyll-containing bacterium capable of degrading biphenyl and dibenzofuran. *Arch. Microbiol*., **178**, 45–52.

Katayama-Fujimura, Y., Komatsu, Y., Kuraishi, H., and Kaneko, T. (1984) Estimation of DNA base composition by high performance liquid chromatography of its nuclease P1 hydrolysate. *Agric. Biol*. *Chem*., **48**, 3169–3172.

Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *J*. *Mol*. *Evol*., **16**, 111–120.

Nishijima, M., Araki-Sakai, M., and Sano, H. (1997) Identification of isoprenoid quinones by frit-FAB liquid chromatography-mass spectrometry for the chemotaxonomy of microorganisms. *J*. *Microbiol*. *Methods*, **28**, 113–122.

Rainey, F. A., Silva, J., Nobre, M. F., Silva, M. T., and da Costa, M. S. (2003) *Porphyrobacter cryptus* sp. nov., a novel slightly thermophilic, aerobic, bacteriochlorophyll *a*-containing species. *Int*. *J*. *Syst*. *Evol*. *Microbiol*., **53**, 35–41.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol*. *Biol*. *Evol*., **4**, 406–425.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **22**, 4673–4680.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, L., Moore, L. H., Moore, W. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P., and Trüper, H. G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacteria systematics. *Int*. *J*. *Syst*. *Bacteriol*., **37**, 463–464.

Yoon, J.-H., Lee, M.-H., and Oh, T.-K. (2004) *Porphyrobacter donghaensis* sp. nov., isolated from sea water of the east sea in Korea. *Int*. *J*. *Syst*. *Evol*. *Microbiol*., **54**, 2231–2235.

Yoon, J.-H., Kang, S.-J., Lee, M.-H., Oh, H. W., and Oh, T.-K. (2006) Porphyrobacter dokdonensis sp. nov., isolated from sea water. Int. J. Syst. Evol. Microbiol., 56, 1079—1083.