Pseudomonas pelagia sp. nov., isolated from a culture of the Antarctic green alga *Pyramimonas gelidicola*

Chung Yeon Hwang,¹ Gwang Il Zhang,¹ Sung-Ho Kang,² Hak Jun Kim² and Byung Cheol Cho¹

Correspondence
Byung Cheol Cho
bccho@snu.ac.kr

¹School of Earth and Environmental Sciences and Research Institute of Oceanography, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea

²Division of Polar Biology and Ocean Sciences, Korea Polar Research Institute, Get-Pearl Tower, Songdo Technopark, 7-50 Songdo-dong, Yeonsu-gu, Incheon 406-840, Republic of Korea

Two Gram-negative, strictly aerobic bacterial strains, designated CL-AP6^T and CL-AP22, were isolated from a culture of a green alga, *Pyramimonas gelidicola*, established from the Antarctic. Cells of the strains were straight rods and motile by means of a single polar flagellum. The strains grew with 0.5–8% (w/v) NaCl (optimum, 1–2%) and at 4–33 °C (optimum, 25 °C) and pH 6.5–9.1 (optimum, pH 7.5–8.1). The two strains shared 98.8% 16S rRNA gene sequence similarity. Analysis of the 16S rRNA gene sequences of strains CL-AP6^T and CL-AP22 revealed that they were members of the genus *Pseudomonas* and were most closely related to *Pseudomonas xiamenensis* C10-2^T (95.5–95.8% sequence similarity) and next to *Pseudomonas pertucinogena* NBRC 14163^T (95.1–95.5% sequence similarity) and to other members of the genus *Pseudomonas* (<95.2% sequence similarity). Phylogenetic analyses based on the 16S rRNA gene sequences showed that strain CL-AP6^T formed a robust clade with strain CL-AP22, and that this clade clustered tightly with the nearest clade containing *P. xiamenensis* and *P. pertucinogena*. The major isoprenoid quinone of strains CL-AP6^T and CL-AP22 was Q-9 and the major cellular fatty acids were C_{18:1ω7c} (40.2–41.6%), summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 26.3–26.8%), C_{16:0} (13.7–13.9%) and C_{12:0} (5.8–6.2%). The genomic DNA G + C contents of strains CL-AP6^T and CL-AP22 were 59.1 and 57.2 mol%, respectively. DNA–DNA hybridization experiments revealed high relatedness values (98.5 ± 0.5%; mean ± SEM, *n* = 2) between strains CL-AP6^T and CL-AP22, indicating that the two strains constituted a single species. However, the two strains differed phenotypically from *P. xiamenensis* by their inability to grow without NaCl, temperature range for growth, hydrolysis of starch and production of certain enzymes. The phylogenetic analysis and physiological and biochemical data showed that strains CL-AP6^T and CL-AP22 should be classified as representing a novel species in the genus *Pseudomonas*, for which the name *Pseudomonas pelagia* sp. nov. is proposed. The type strain is CL-AP6^T (=KCCM 90073^T = JCM 15562^T).

Since the genus *Pseudomonas* was proposed by Migula (1894), members of novel *Pseudomonas* species have been isolated from many natural materials including soil, fresh and saline waters, plants and clinical specimens (Palleroni, 2005). Because of its broad phenotypic definition (i.e.

polarly flagellated, Gram-negative, rod-shaped, aerobic bacteria; Palleroni, 1984), the genus *Pseudomonas* is an extensively heterogeneous group (Gupta *et al.*, 2008). The genus has been divided into distinct groups several times on the basis of phenotypic features (Sneath *et al.*, 1981), DNA–DNA hybridization (Palleroni, 1984), chemotaxonomic data (Oyaizu & Komagata, 1983) and 16S rRNA gene sequence similarity (Anzai *et al.*, 2000). In *Bergey's Manual of Systematic Bacteriology* (Palleroni, 2005), 53 species and 8 putative species of the genus *Pseudomonas* have been described. Thereafter, about 60 species of the genus *Pseudomonas* were discovered. Based on 16S rRNA gene sequences, the genus appears to consist of two major

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CL-AP6^T and CL-AP22 are EU888911 and FJ687951, respectively.

An extended neighbour-joining tree based on 16S rRNA gene sequences showing the positions of strains CL-AP6^T and CL-AP22 and related *Pseudomonas* species, and a transmission electron micrograph of a negatively stained cell of strain CL-AP6^T are available as supplementary material with the online version of this paper.

clusters (Anzai *et al.*, 2000). The vast majority (>95 %) of recognized species or subspecies of the genus *Pseudomonas* are included in one of the two major clusters. In the other cluster, the *Pseudomonas pertucinogena* group includes only two species with validly published names; *Pseudomonas pertucinogena* (Kawai & Yabuuchi, 1975) and *Pseudomonas xiamenensis* (Lai & Shao, 2008). In the present study, two strains affiliated with the *P. pertucinogena* group were isolated from a culture of an Antarctic green alga and subjected to a polyphasic taxonomic analysis.

A culture of the green alga *Pyramimonas gelidicola* (Korea Polar Research Institute, KOPRI Culture Collection of Polar Micro-organism, KCCPM strain no. AnM0046) was established from the Antarctic Ocean in 1981. It was maintained by transferring small quantities of the culture to fresh f/2 medium (Guillard & Ryther, 1962) once a month. The culture was incubated at 2–4 °C under continuous light conditions. For the isolation of psychrophilic or psychrotolerant bacteria, an aliquot (10 µl) of the culture was spread on two marine agar 2216 (MA; Difco) plates in a cold room (4 °C). The plates were incubated aerobically at 4 °C in the light for 2 weeks. Strain CL-AP6^T was isolated and subsequently streaked onto fresh MA at 4 °C. The purification procedure was repeated four times. Strain CL-AP22 (=KCCM 90081) was isolated from the same culture of *Pyramimonas gelidicola* using the above isolation and purification procedure on a different day. The strains were maintained both on MA at 4 °C and in marine broth 2216 (MB; Difco) supplemented with 30 % (v/v) glycerol at –80 °C.

For 16S rRNA gene amplification by PCR, DNA was extracted from a single colony by using a boiling method (Englen & Kelley, 2000). The crude extracts served as the DNA template for PCR, which included *Taq* DNA polymerase (Bioneer) and primers 27F and 1492R (Lane, 1991). The PCR product was purified by using an *AccuPrep* PCR purification kit (Bioneer) and direct sequence determination of the purified 16S rRNA gene was performed using sequencing primers (27F, 518F, 800R and 1492R; Lane, 1991; Anzai *et al.*, 1997) with an Applied Biosystems automated sequencer (ABI3730XL) at Macrogen, Seoul, Korea. Almost-complete 16S rRNA gene sequences of strains CL-AP6^T (1460 bp) and CL-AP22 (1415 bp) were obtained and compared with 16S rRNA gene sequences available in GenBank using BLASTN searches (Altschul *et al.*, 1990). The sequences of strains CL-AP6^T and CL-AP22 were aligned with sequences from the type strains of some *Pseudomonas* species, obtained from GenBank and Ribosomal Database Project II (Cole *et al.*, 2007) databases, using known 16S rRNA gene secondary-structure information. Phylogenetic trees were obtained by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of the tree topologies was assessed

by bootstrap analyses based on 1000 replications for neighbour-joining and maximum-parsimony methods. Alignment analysis was carried out using the jPHYDIT program (Jeon *et al.*, 2005) and phylogenetic analyses were carried out using MEGA 4 (Tamura *et al.*, 2007) and PAUP 4.0 (Swofford, 1998). Likelihood parameters were estimated by using the hierarchical ratio test in MODELTEST, version 3.04 (Posada & Crandall, 1998) and bootstrap analysis based on 100 replications was conducted. The DNA–DNA relatedness between strains CL-AP6^T and CL-AP22 was determined by using dot-blot hybridization as described by Choi *et al.* (2006). The experiment was repeated on different days.

Analysis of the 16S rRNA gene sequences of strains CL-AP6^T and CL-AP22 revealed that they shared 98.8 % similarity. They were most closely related to *P. xiamenensis* C10-2^T (95.5–95.8 % sequence similarity) and next to *P. pertucinogena* NBRC 14163^T (95.1–95.5 % sequence similarity). The sequence similarities of the two strains to other recognized species of the genus *Pseudomonas* were below 95.2 %. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains CL-AP6^T and CL-AP22 belonged to the genus *Pseudomonas* (Fig. 1). In addition, strain CL-AP6^T formed a robust clade with strain CL-AP22, and this clade clustered tightly with the nearest clade comprising *P. xiamenensis* and *P. pertucinogena* (Fig. 1). This grouping was supported by high bootstrap values (neighbour-joining, 99 %; maximum-likelihood, 91 %; and maximum-parsimony, 93 %). DNA–DNA hybridization experiments revealed high relatedness values (98.5 ± 0.5 %; mean $\pm$ SEM, $n=2$) between strains CL-AP6^T and CL-AP22, indicating that they belonged to the same species. Thus, the low similarity value (i.e. <96 %) with recognized *Pseudomonas* species and the phylogenetic positions of strains CL-AP6^T and CL-AP22 indicated that they represent a novel species of the genus *Pseudomonas*.

Growth rates (approximately 2.4 d^{–1}) of strains CL-AP6^T and CL-AP22 in Luria–Bertani broth (LB; Sambrook & Russell, 2001) at 25 °C were comparable to those in MB at 25 °C (data not shown). In addition, both strains grew well on LB agar as well as on MA. Unless otherwise specified, all tested physiological and biochemical characteristics of the two strains were based on cultures grown on LB agar at 25 °C with *P. xiamenensis* JCM 13530^T and *P. pertucinogena* JCM 11590^T as reference strains, except for susceptibility to antibiotics, and fatty acid and quinone analyses. Morphological and physiological analyses were performed as follows. Gram-staining was performed as described by Smibert & Krieg (1994). Motility of the cells was observed by using the hanging drop method (Skerman, 1967). Cell morphology and the presence of flagella were observed using transmission electron microscopy (EX2; JEOL). Anaerobic growth was checked on LB agar and a modified ZOF medium (per litre distilled water: tryptone, 10 g; yeast extract, 5 g; glucose, 10 g; phenol red, 10 mg; Bacto agar, 15 g; Lemos *et al.*, 1985) by using the GasPak anaerobic system (BBL) for 15 days. Poly-β-hydroxybutyrate gran-

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strains CL-AP6^T, CL-AP22 and type strains of some related *Pseudomonas* species based on 16S rRNA gene sequences. Only bootstrap values above 70% are shown (1000 resamplings) at branch points. Solid circles indicate that the corresponding nodes were also obtained in maximum-likelihood and maximum-parsimony trees. *Halomonas elongata* was used as an outgroup. Bar, 0.01 nucleotide substitutions per site. The full version of this phylogenetic tree is available as Supplementary Fig. S1 (in IJSEM Online).

ules were observed by using epifluorescence microscopy (BX60; Olympus) after Nile blue A staining (Ostle & Holt, 1982). Endospores were detected by using malachite green staining (Smibert & Krieg, 1994). The formation of fluorescent pigments was tested on King's medium B (King *et al.*, 1954) with *Pseudomonas koreensis* KACC 10848^T and *Pseudomonas jinjuensis* KACC 10760^T as a positive and a negative strain, respectively (Kwon *et al.*, 2003). The temperature range for growth was determined on the basis of colony formation on LB agar incubated at temperatures ranging from 5 to 40 °C (increments of 5 °C) and 4, 33 and 37 °C. The pH range (pH 5.1–10.2; pH 5.1, 5.9, 6.5, 7.2, 7.5, 8.1, 8.4, 9.1, 9.6 and 10.2) for growth was determined by assessing changes in OD₆₀₀ over the incubation period (up to 7 days) in LB broth at 25 °C. Prior to autoclaving, the pH (5–10, in increments of 0.5 pH units) was adjusted using 1 M NaOH and 1 M HCl solutions. After autoclaving and cooling, the pH values for the subsamples of each medium were measured, before the inoculation of cells. The tolerance of NaCl was determined by assessing changes in OD₆₀₀ in LB medium supplemented with NaCl concentrations of 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 12% (w/v) at 25 °C.

Cells of strains CL-AP6^T and CL-AP22 were Gram-negative, strictly aerobic and motile by means of a single polar flagellum (Supplementary Fig. S2, available in IJSEM Online). Strains CL-AP6^T and CL-AP22 could be differentiated from the phylogenetically closely related species (i.e. *P. xiamenensis* JCM 13530^T and *P. pertucinogena* JCM 11590^T) by their ability to grow at 4 °C and their inability to grow at 37 °C (Table 1). In addition, the two strains were not able to grow without NaCl, whereas *P.*

xiamenensis JCM 13530^T and *P. pertucinogena* JCM 11590^T were able to grow without NaCl (Table 1). The results of the other physiological and biochemical tests are given in the species description and in Table 1.

Catalase and oxidase activities and H₂S production were assayed according to the protocols described by Smibert & Krieg (1994). Amylase, gelatinase, nitrate reductase activities and degradation of Tweens 20, 40, 60 and 80, and Voges–Proskauer and methyl red tests were determined according to Hansen & Sørheim (1991) using LB instead of MB as growth medium. Other enzyme activities were assayed using the API ZYM and API 20NE kits (bioMérieux) according to the manufacturer's instructions. To examine sole carbon utilization, a basal broth medium with the NaCl concentration adjusted to 1% (Liu & Shao, 2005) containing 0.1% carbon source was used. Growth was monitored by OD₆₀₀ using a spectrophotometer (Ultraspec 2000; Pharmacia Biotech). OD₆₀₀ was measured for 20 days. Carbon utilization was scored as negative when growth rate was equal to, or less than that in the negative control with no carbon source. Growth rate was measured by assessing changes in OD₆₀₀. Resistance to antibiotics was determined by using the disc diffusion plate method (Bauer *et al.*, 1966).

Strains CL-AP6^T and CL-AP22 showed almost identical results for phenotypic characteristics except that naphthol-AS-BI-phosphohydrolase activity was present in strain CL-AP6^T but not in strain CL-AP22 (Table 1). Strains CL-AP6^T and CL-AP22 could be distinguished from *P. xiamenensis* and *P. pertucinogena* by means of the following traits: the ability to hydrolyse starch and the presence of

Table 1. Differential characteristics of strains CL-AP6^T and CL-AP22 and other phylogenetically related *Pseudomonas* species

Taxa: 1, strains CL-AP6^T and CL-AP22 (data from this study); 2, *P. xiamenensis* C10-2^T (Lai & Shao, 2008); 3, *P. pertucinogena* ATCC 190^T (Kawai & Yabuuchi, 1975). +, Positive; –, negative; w, weakly positive.

Characteristic	1*	2	3
Cell size (µm)	0.4–0.5 × 1.8–2.0	0.6–0.8 × 1.1–1.3	0.4 × 1.1
Growth at:			
4 °C	+	–	–†
37 °C	–	+	+
NaCl range for growth (%)	0.5–8	0–8	0–5‡
NaCl range for optimal growth (%)	1–2	1–3	3
Nitrate reduction	+	+	–
Hydrolysis of:			
Starch	+	–	–
Tween 80	–	+	–
API ZYM†			
N-Acetyl-β-glucosaminidase	–	+	–
Acid phosphatase	+	w	–
Alkaline phosphatase	+	w	–
α-Chymotrypsin	–	+	–
Esterase (C4)	+	–	–
Esterase lipase (C8)	+	+	–
β-Galactosidase	–	+	–
Lipase (C14)	+	–	–
Naphthol-AS-BI-phosphohydrolase	+	+	–
Trypsin	–	+	–
Valine arylamidase	–	+	–
Utilization of sole carbon source			
Citrate	–	+	–‡
Ethanol	–	+	–
Succinate	–	–	+
DNA G + C content (mol%)	59.1 (57.2)	61.2	60.0

*Differential data for strain CL-AP22 are shown in parentheses; otherwise, congruent results were obtained for both strains.

†Data from this study.

‡Data from Lai & Shao (2008).

esterase (C4) and lipase (C14) activities. Furthermore, the inability to hydrolyse Tween 80 and the absence of *N*-acetyl-β-glucosaminidase, α-chymotrypsin, β-galactosidase, trypsin and valine arylamidase activities, and the inability to utilize citrate or ethanol as a sole carbon source differentiated strains CL-AP6^T and CL-AP22 from *P. xiamenensis* (Table 1). The two strains could be differentiated from *P. pertucinogena* by the ability to reduce nitrate, the presence of acid and alkaline phosphatases and esterase lipase (C8) activities, and the inability to utilize succinate as a sole carbon source (Table 1).

The fatty acid methyl esters of whole cells of strains CL-AP6^T and CL-AP22 were analysed by gas chromatography according to the instructions of the Microbial Identification System (MIDI) at the Korean Culture Center of Micro-organisms (KCCM) in Seoul, Korea. The strains were grown in LB broth at 28 °C for 2 days. 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 by the method of Marmur (1961). Lambda phage DNA was used as a standard. The isoprenoid quinone composition was determined according to Minnikin *et al.* (1984) and analysed by HPLC as described by Collins (1985), using *Pseudomonas chlororaphis* ATCC 9446^T (Yamada *et al.*, 1982) as a reference strain.

The fatty acid profiles were similar between strains CL-AP6^T and CL-AP22 (Table 2): the major fatty acids of the two strains were C_{18:1ω7c} (40.2–41.6%), summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 26.3–26.8%), C_{16:0} (13.7–13.9%) and C_{12:0} (5.8–6.2%). Other fatty acids are shown in Table 2. The fatty acids C_{18:1ω7c} and C_{16:0} were found predominantly in strains CL-AP6^T and CL-AP22 and related *Pseudomonas* species (Table 2). However, a significant amount of summed feature 3 was found only in strains CL-AP6^T and CL-AP22. Furthermore, the absence of fatty acid C_{16:1ω9c} clearly differentiated strains CL-AP6^T and CL-AP22 from the related *Pseudomonas* species

Table 2. Cellular fatty acid contents of strains CL-AP6^T and CL-AP22 and other phylogenetically related *Pseudomonas* species

Taxa: 1, strains CL-AP6^T and CL-AP22 (data from this study); 2, *P. xiamenensis* C10-2^T (Lai & Shao, 2008); 3, *P. pertucinogena* JCM 11590^T (Lai & Shao, 2008). Unless otherwise indicated, all strains were cultured in LB broth at 28 °C for 2 days. ND, Not detected; tr, trace amount (<1 %).

Fatty acid	1		2	3
	LB*	TSA†		
Straight-chain fatty acids				
C _{12:0}	6.2 (5.8)	7.3	2.3	4.6
C _{14:0}	tr (tr)	tr	4.7	tr
C _{15:0}	tr (tr)	tr	2.0	tr
C _{16:0}	13.7 (13.9)	8.4	32.5	18.8
C _{17:0}	tr (tr)	tr	1.6	tr
C _{18:0}	1.0 (1.2)	tr	5.5	8.8
C _{17:0} cyclo	1.7 (2.1)	3.8	ND	ND
Unsaturated fatty acids				
C _{15:1}	ND (ND)	ND	1.0	tr
C _{17:1}	ND (ND)	ND	2.5	tr
C _{19:1}	ND (ND)	ND	1.2	1.7
C _{16:1} ω9c	ND (ND)	ND	17.7	8.0
C _{18:1} ω7c	41.6 (40.2)	40.8	17.5	52.9
C _{18:1} ω9c	ND (ND)	ND	6.4	1.4
Hydroxy fatty acids				
C _{10:0} 3-OH	1.8 (1.4)	2.8	ND	ND
C _{12:0} 3-OH	3.2 (2.8)	3.6	tr	tr
C _{16:0} 3-OH	ND (ND)	ND	3.6	tr
Summed feature 3‡	26.3 (26.8)	28.1	ND	ND

*Data in parentheses were for strain CL-AP22.

†Data were from a different culture condition (TSA with 2 %, w/v, NaCl at 25 °C for 2 days).

‡Summed feature 3 contains C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

(Table 2). The major isoprenoid quinone of strains CL-AP6^T and CL-AP22 was Q-9. The genomic DNA G+C contents of strains CL-AP6^T and CL-AP22 were 59.1 and 57.2 mol%, respectively. These data support the attribution of strains CL-AP6^T and CL-AP22 to the genus *Pseudomonas* (Palleroni, 2005).

Thus, on the basis of the data from the polyphasic taxonomic study, strains CL-AP6^T and CL-AP22 should be placed in the genus *Pseudomonas* as representing a novel species, for which the name *Pseudomonas pelagia* sp. nov. is proposed.

Description of *Pseudomonas pelagia* sp. nov.

Pseudomonas pelagia (pe.la'gi.a. L. fem. adj. *pelagia* of the sea).

Gram-negative, strictly aerobic and motile by a single polar flagellum. Cells are non-spore-forming, straight rod-

shaped, approximately 0.4–0.5 µm in width and 1.8–2.0 µm in length. Cells do not contain poly-β-hydroxybutyrate granules. No fluorescent pigments are detected. H₂S is not produced. After 3 days on LB agar at 25 °C, colonies are circular, convex and white in colour, and about 1.5–2 mm in diameter. Cells also grow on MA and tryptic soy agar (TSA; Difco). Growth occurs within the temperature range 4–33 °C (optimum, 25 °C) and at pH 6.5–9.1 (optimum, pH 7.5–8.1). Growth occurs in NaCl concentrations of 0.5–8 %, w/v (optimum, 1–2 %). Oxidase and catalase are positive. Positive for amylase and nitrate reductase (a congruent result was obtained in the API 20NE test) activities and degradation of Tweens 20, 40 and 60. Negative for gelatinase activity (a congruent result was obtained in the API 20NE test), hydrolysis of Tween 80, and Voges–Proskauer and methyl red tests. According to API ZYM tests, acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), lipase (C14) and leucine arylamidase activities are positive, but *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, α-mannosidase, trypsin and valine arylamidase activities are negative, and naphthol-AS-BI-phosphohydrolase activity is variable. According to the API 20NE tests, arginine dihydrolase, aesculin hydrolysis, β-galactosidase, glucose fermentation, indole production and urease are negative. D-Glucose, peptone and pyruvate are utilized as a sole carbon source, but acetate, *N*-acetyl-D-glucosamine, L-arginine, aspartic acid, ascorbate, ethanol, citrate, D-fructose, D-galactose, L-glutamate, glycerol, inositol, lactose, D-mannose, D-ribose, succinate and sucrose are not utilized. Cells are sensitive to (µg per disc): chloramphenicol (20), gentamicin (6), kanamycin (20) and polymyxin B (25) but resistant to ampicillin (6), cephalixin (20), erythromycin (10), mitomycin C (0.6), nalidixic acid (20), penicillin G (6), streptomycin (6), tetracycline (20) and vancomycin (20). The isoprenoid quinone is Q-9. Major cellular fatty acids are C_{18:1}ω7c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{16:0} and C_{12:0}. The DNA G+C content of the type strain is 59.1 mol%.

The type strain, CL-AP6^T (=KCCM 90073^T=JCM 15562^T), was isolated from a culture of the Antarctic green alga *Pyramimonas gelidicola*. Strain CL-AP22 (=KCCM 90081), isolated from the same source, is a second strain of the species.

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