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Photobacterium profundum sp. nov., a new, moderately barophilic bacterial species isolated from a deep-sea sediment

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Abstract A novel, moderately barophilic bacterium was isolated from a sediment sample obtained from the Ryukyu Trench, at a depth of 5110 m. The isolate, designated strain DSJ4, is a Gram-negative rod capable of growth between 4°C and 18°C under atmospheric pressure, with optimum growth displayed at 10°C, and capable of growth at pressures between 0.1 MPa and 70 MPa at 10°C, with optimum growth displayed at 10 MPa. Strain DSJ4 is a moderately barophilic bacterium, and shows no significant change in growth at pressures up to 50 MPa. Phylogenetic analysis of the 16S rRNA sequence of strain DSJ4 places this strain within the *Photobacterium* subgroup of the family *Vibrionaceae*, closely related to the strain SS9 that was independently isolated from the Sulu Trough. The temperature and pressure ranges for growth, cellular fatty acid composition, and assorted physiological and biochemical characteristics indicate that these strains differ from other *Photobacterium* species. Furthermore, both SS9 and DSJ4 displayed a low level of DNA similarity to other *Photobacterium* type strains. Based on these differences, these strains are proposed to represent a new deep-sea-type species. The name *Photobacterium profundum* (JCM10084) is proposed.

Key words Barophilic bacterium · Deep sea · Hydrostatic pressure · *Photobacterium* · Ryukyu Trench

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Introduction

Numerous deep-sea barophilic bacterial strains have been isolated and characterized since the 1979 report by Yayanos et al. (1979), in an effort to understand the interaction between the deep-sea environment and its microbial inhabitants. Barophilic bacteria are characterized by enhanced growth at pressures above 1 atmosphere. Thus far, all barophilic bacterial isolates fall into the Proteobacteria γ-subgroup according to phylogenetic classifications derived from 16S ribosomal RNA sequence information (Kato et al. 1995; DeLong et al. 1997). DeLong et al. (1997) reported that eleven cultivated psychrophilic and barophilic deep-sea bacteria are affiliated with one of five genera within the γ-subgroup: *Shewanella, Photobacterium*, *Colwellia*, *Moritella*, and a new group. The deep-sea-adapted type species of three of these genera were previously reported as *S. benthica* in the genus *Shewanella* (Deming et al. 1984), *C. hadaliensis* in the genus *Colwellia* (Deming et al. 1988), and *M. marinus* in the genus *Moritella* (Colwell and Morita 1964; Steven 1990).

Members of the genus *Photobacterium* are common bacteria in marine environments (Ruby and Nealson 1978). *Photobacterium* sp. strain SS9, isolated from the Sulu Trough associated with Amphipoda at a depth of 2551 m, was the first reported deep-sea *Photobacterium* species (DeLong 1986; DeLong et al. 1997). Strain SS9 is a moderately barophilic strain and its mechanisms of high-pressure adaptation have been well studied at the molecular and genetic level by Bartlett and his co-workers (Bartlett et al. 1989, 1996). Although the 16S rRNA sequence of strain SS9 has recently been obtained (DeLong et al. 1997), additional information relating to its taxonomic placement within the genus *Photobacterium* is needed. Because the genus *Photobacterium* may be one of the dominant cultivatable benthic bacterial groups, their taxonomic characterization is an important component of the characterization of deep-sea microbial populations.

In this paper, we describe the properties of strain DSJ4 isolated from a deep-sea sediment sample recovered from

the Ryukyu Trench at a depth of 5110 m, and demonstrate that this strain also belongs to the genus *Photobacterium*. Several lines of evidence indicate that DSJ4 and SS9 are members of the same species. Our results suggest that these strains should be classified as a new species, proposed as *Photobacterium profundum*, and herein present its description.

Materials and methods

Sample collection

Sediment samples used for isolating deep-sea adapted microorganisms were collected from the Ryukyu Trench (5110 m deep; 24°15.23′N, 126°47.30′E) by means of sterilized mud samplers using the manned submersible *Shinkai 6500* operated by the Japan Marine Science and Technology Center (Takagawa et al. 1989; Kato et al. 1995).

Bacterial strains and culture conditions

The deep-sea bacterium DSJ4 was isolated according to the procedure described previously (Kato et al. 1995). The reference strains used in this study, *Photobacterium angustum* ATCC 25915^T (T = type strain), *Photobacterium damselae* ATCC 33539^T, *Photobacterium leiognathi* ATCC 25521^T, and *Photobacterium phosphoreum* ATCC 11040^T were obtained from the American Type Culture Collection (Rockville, MD, USA). *Photobacterium histaminum* JCM 8968^T was obtained from the Japan Collection of Microorganisms (Wako-shi, Saitama, Japan). *Photobacterium* sp. strain SS9 was kindly provided by Drs. A.A. Yayanos and D.H. Bartlett (Scripps Institution for Oceanography, UCSD, La Jolla, CA, USA). These bacteria were maintained on Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA). The temperature for cultivation of these strains was 20°C except for strains DSJ4 and SS9 which were grown at 10°C and 15°C, respectively. High pressure cultivation was performed according to the procedure reported previously (Kato et al. 1995).

Microscopy

The morphologies of living and nonliving stained cells were determined by light microscopy and transmission electron microscopy, respectively. For negative staining, one drop of a culture was placed on a copper grid coated with Pioloform and carbon and stained with 1% potassium phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. The negatively-stained cells were observed with a model JEM-1210 transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

Cellular fatty acids and isoprenoid quinones

Cells were grown in Marine Broth 2216 under atmospheric pressure for 1 day, washed twice with 0.7% NaCl solution at 4°C by centrifugation at 8000 × *g*, and freeze-dried. The dried cells (20 mg) were placed in a Teflon-lined, screw-capped tube containing 2 ml of anhydrous methanolic HCl and heated at 100°C for 3 h. After cooling, 1 ml of water was added, and the fatty-acid methyl esters were extracted with *n*-hexane. The samples were analyzed for cellular fatty-acids using a gas-liquid chromatography-mass spectrometer (Komagata and Suzuki 1987).

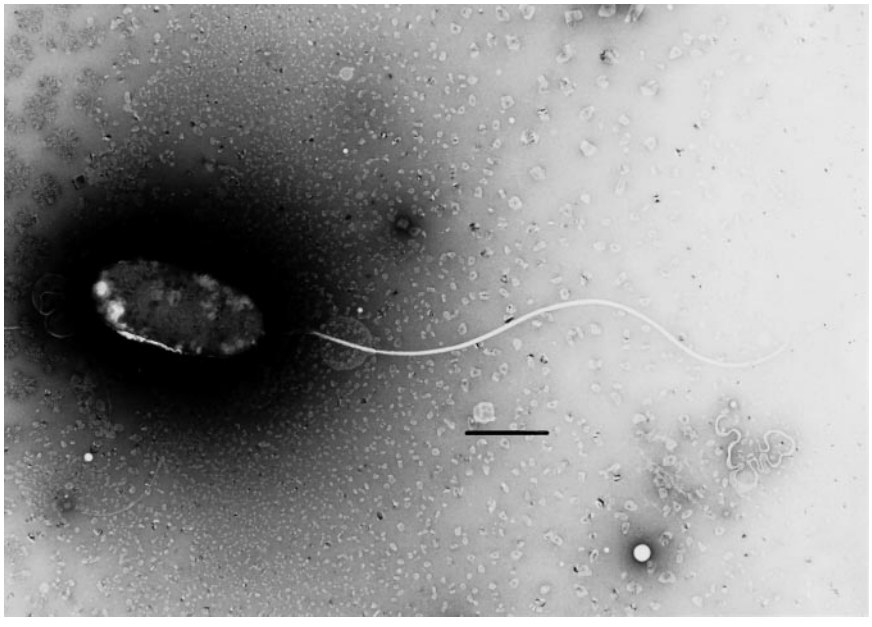

Fig. 1. Transmission electron microscopy of a negatively-stained cell of strain DSJ4. *Bar*, 1 μm

The isoprenoid quinones were extracted with chloroform-methanol (2:1) from dried cells (200 mg) and were purified by thin-layer chromatography. The purified isoprenoid quinones were analyzed by reverse-phase high-performance liquid chromatography (Komagata and Suzuki 1987).

DNA studies

DNA was extracted by the method of Saito and Miura (1963). The guanine-plus-cytosine (G + C) content was determined by reverse-phase high-performance liquid chromatography (Tamaoka and Komagata 1984). For analysis of relatedness, DNA–DNA hybridization was carried out at 40°C for 3 h and measured fluorometrically by the method of Ezaki et al. (1989).

Sequence analysis of the 16S rRNA gene and phylogenetic tree construction

Polymerase chain reaction (PCR) amplification of the 16S rRNA gene (16S rDNA) was performed with a DNA Thermal Cycler model 9600 (Perkin-Elmer/Cetus, Norwalk, CT, USA) using 50 µl of PCR reaction mixture in each instance under the conditions recommended by the enzyme manufacturer (Takara, Otsu, Japan) according to the procedure reported previously (Kato et al. 1997). PCR-amplified DNAs were analyzed by electrophoresis through a 1.5% agarose gel. DNA sequences were determined using an automated DNA sequencer model 373S (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA) according to the procedure described previously (Kato et al. 1977).

Nucleotide substitution rates (K_{nuc}) (Kimura 1980) were determined, and a distance matrix tree was constructed by the neighbor-joining method (Saitou and Nei 1987), using the CLUSTAL W program (Thompson et al. 1994). Alignment gaps and unidentified base positions were not taken into consideration for the calculations. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 bootstrapped trials.

Nucleotide sequence accession numbers

The 16S rDNA sequence of *Photobacterium profundum* sp. strain DSJ4 JCM 10084, determined in this study, has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D21226. The accession numbers for the sequences used as references are as follows: *Aeromonas hydrophila* ATCC 7966^T, X74677; *Aeromonas veronii* ATCC 35624^T, X74684; *Escherichia coli*, V00348; *Listonella anguillarum*, ATCC 19264^T, X16895; *Photobacterium angustum* ATCC 25915^T, D25307; *Photobacterium damselae* ATCC 33539^T, X74700; *Photobacterium fischeri* ATCC 7744^T, X74702; *Photobacterium histaminum* JCM 8968^T, D25308; *Photobacterium leiognathi* ATCC 25521^T, D25309; *Photobacterium phosphoreum* ATCC 11040^T, D25310; *Photobacterium* sp. strain SS9, AB003191; *Vibrio alginolyticus* ATCC 17749^T, X74690; *Vibrio campbellii* ATCC 25920^T, X74692; *Vibrio cholerae* ATCC 14035^T, X74694; *Vibrio diazotrophicus* ATCC 33466^T, X74701; *Vibrio harveyi* ATCC 14126^T, X74706; *Vibrio metschnikovii* CIP 69.14^T, X74712; *Vibrio nereis* ATCC 25917^T, X74716; and *Vibrio parahaemolyticus* ATCC 17802^T, X74721. *Bacillus subtilis*, X60646, was included as an outgroup.

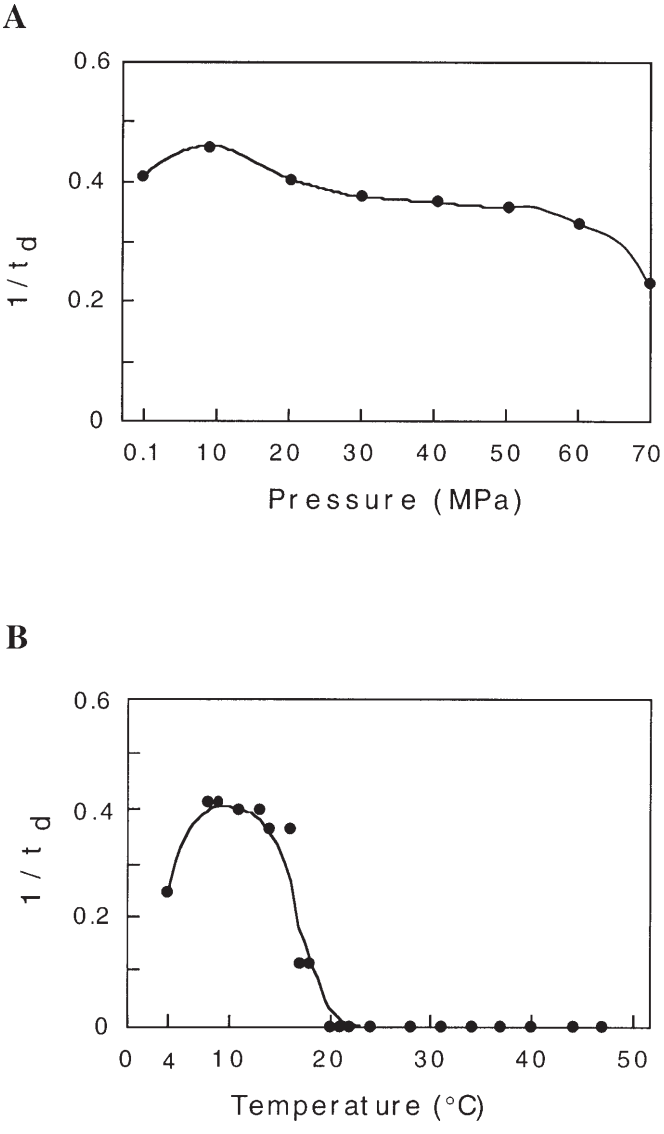

Fig. 2. Growth profiles of strain DSJ4 at several temperatures (**A**) and pressures (**B**). t_d, doubling time (h)

Results and discussion

Morphology and growth properties

Cells of the isolated deep-sea strain DSJ4 were Gram-negative, rods, 2–4 µm long and 0.8–1.0 µm wide (Fig. 1),

Table 1. Characteristics of the strain DSJ4 and *Photobacterium* reference strains

Characteristics	*P. angustum* ATCC 25915^T	*P. damselae* ATCC 33539^T	*P. histaminum* JCM 8968^T	*P. leiognathi* ATCC 25521^T	*P. phosphoreum* ATCC 11040^T	*Photobacterium* sp. SS9	Strain DSJ4
Quinone	Q-8	Q-8(90%) Q-7(10%)	Q-8(90%) Q-7(10%)	Q-8(90%) Q-7(10%)	Q-8	Q-8	Q-8(95%) Q-7(5%)
Optimum growth temperature (°C)	25	26	26	26	18	15	10
Optimum growth pressure (MPa)	0.1	0.1	0.1	0.1	0.1	20	10
Motility	+	+	+	+	+	+	+
O/F test	F	F	F	F	F	F	F
Gas produced with growth on glucose	−	+	−	+	+	w	−
Catalase	−	+	+	−	+	+	+
Oxidase	−	+	+	+	+	+	+
Production of H_2S	−	−	−	−	−	−	−
Production of indole	−	−	−	−	−	+	+
Nitrate reduced	−	+	+	+	+	+	+
Nitrite reduced	−	−	−	−	−	−	−
O/129 sensitivity:							
10 μg	+	+	−	+	+	+	−
150 μg	+	+	+	+	+	+	−
Arginine dihydrolase	+	+	+	+	+	+	+
Lysine decarboxylase	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	−	−	−	−
Glutamine decarboxylase	−	−	−	+	−	−	−

+, positive; −, negative; w, weakly positive; Q, quinone; O, oxidation; F, fermentation; O/129, 2,4-diamino-6,7-diisopropyl pteridine.

Table 2. Carbon utilization by strain DSJ4 and *Photobacterium* reference strains

Carbon sources	*P. angustum* ATCC 25915^T	*P. damselae* ATCC 33539^T	*P. histaminum* JCM 8968^T	*P. leiognathi* ATCC 25521^T	*P. phosphoreum* ATCC 11040^T	*Photobacterium* sp. SS9	Strain DSJ4
Glycogen	+	+	+	+	+	+	+
Tween 40	−	+	−	−	+	+	+
Tween 80	−	−	−	−	−	+	+
N-acetyl-D-galactosamine	+	+	+	+	−	−	−
N-acetyl-D-glucosamine	+	+	+	+	+	+	−
Adonitol	−	−	−	−	−	−	−
L-arabinose	−	−	−	−	−	−	−
Cellobiose	−	+	+	−	−	+	−
D-fructose	+	+	+	+	+	+	−
D-galactose	+	+	+	+	−	+	+
α-D-glucose	+	+	+	+	+	+	+
Myo-inositol	−	−	−	−	−	+	−
α-D-lactose	−	−	−	−	−	−	−
Maltose	+	+	+	+	+	+	+
D-mannitol	−	−	−	−	−	+	+
D-mannose	+	+	+	+	+	+	+
D-raffinose	−	−	−	−	−	−	−
L-rhamnose	−	−	−	−	−	−	−
D-sorbitol	−	−	−	−	−	−	−
Sucrose	+	−	−	−	−	−	−
D-trehalose	−	−	+	−	−	+	+
Turanose	+	+	+	+	−	−	−
Glycerol	+	+	+	+	−	+	+

+, positive; −, negative.

which were motile by means of a single unsheathed polar flagellum. Strain DSJ4 was able to grow in pressure vessels under hydrostatic pressures in the range of 0.1–70 MPa and at temperatures in the range of 4°–18°C (Fig. 2). The optimal temperature and pressure conditions for growth were 8°–12°C and 10 MPa, respectively. This strain was not able to grow at temperatures above 20°C. These findings suggest that isolate DSJ4 is a moderately barophilic and psychrophilic bacterium.

Biochemical and physiological characteristics

Characteristics of strain DSJ4 are shown in Tables 1 and 2. This strain is a facultatively anaerobic chemoorganotroph

having both respiratory and fermentative types of metabolism. Catalase and cytochrome oxidase are positive. Acids are produced from D-glucose but gas is not produced. Nitrate is reduced to nitrite. It is negative for H_2S production and is sensitive to the vibriostatic agent 2,4-diamino-6,7-diisopropyl pteridine (O/129). The following compounds are utilized as sole carbon and energy sources: glycogen, Tween 40, Tween 80, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, D-trehalose, and glycerol. The following compounds are not utilized: *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, cellobiose, D-fructose, myo-inositol, lactose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, and turanose. Among the reference strains, *Photobacterium histaminum* shares the most physiological characteristics with DSJ4. However, the properties of indole production and optimum growth temperature differ, as shown in Table 1. Other differences in properties between the standard *Photobacterium* species and strain DSJ4 are as follows. Strain DSJ4 was able to utilize Tween 80 and D-mannitol but not able to utilize *N*-acetyl-D-galactosamine or turanose. No growth occurred above 20°C, and its optimum growth temperature at atmospheric pressure was 10°C. Strains DSJ4 and SS9 are moderately barophilic strains and able to grow at 50 MPa, but the reference species were not able to grow under such high-pressure conditions (data not shown). Strain DSJ4 was not able to grow without NaCl in the medium, and the optimal concentration of NaCl was around 3% (data not shown).

Fatty acid composition and isoprenoid quinones

The whole-cell fatty acid composition of strain DSJ4 and that of selected reference strains are shown in Table 3. The dominant fatty acid in DSJ4 was hexadecenoic acid (16:1), and the other fatty acids present in significant amounts were 14-methylpentadecanoic acid (iso-16:0), hexadecanoic acid (16:0), octadecenoic acid (18:1), and icosapentaenoic acid (IPA) (20:5). The fatty acid profile of strain DSJ4 was very different compared with the reference species and this strain was particularly characterized by containing substantial amounts of iso-16:0 and 20:5. The fatty acid composition of strain DSJ4 was similar to that of the other deep-sea strain tested, SS9.

The occurrence of polyunsaturated fatty acid (20:5 ω3c, IPA) is a property of psychrophilic and some deep-sea adapted bacteria. For example, the deep-sea *Shewanella* species *Shewanella benthica* sp. PT99 produces IPA, but the shallow species *Shewanella putrefaciens* does not produce IPA (DeLong et al. 1997). Thus, the existence of such fatty acids may be used to define deep-sea species.

The major isoprenoid quinone of strain DSJ4 was Q-8 and a minor quinone was Q-7, which accounted for 95% and 5% of the total isoprenoid quinones, respectively (Table 1).

DNA–DNA hybridization

The results of DNA–DNA hybridization analysis comparing the deep-sea strains DSJ4 and SS9, and the *Photobacterium* reference strains are shown in Table 4. The hybridization values obtained between DSJ4 or SS9 and the *Photobacterium* reference strains were significantly lower than that accepted as the phylogenetic definition of a species (Wayne et al. 1987). However, a high level of DNA–DNA relatedness between the deep-sea strains, DSJ4 and SS9, was obtained (78%), thus indicating that these strains are probably members of the same species (Wayne et al. 1987). Other results in Tables 1 and 3 also tend to support this conclusion.

Table 3. Fatty acid compositions of strain DSJ4 and *Photobacterium* reference strains

Strains	% of total fatty acids																
	12:0	12:0 3-OH	13:0 iso	14:0 iso	14:0	14:1	14:0 3-OH	15:0 iso	15:0	16:0 iso	16:0	16:1	17:0	17:1	18:0	18:1	20:5 (ω3c)
P. angustum ATCC 25915^T	6	8			3	1	3		2		19	36	3	1	2	17	
P. damselae ATCC 33539^T	4	6			5	2	2		3		20	31	2	1	2	23	
P. histaminum JCM 8968^T	3	6			9	1	2				25	44				9	
P. leiognathi ATCC 25521^T	5	6			7	1	2		2		24	34	2	1	2	15	
P. phosphoreum ATCC 11040^T	6	9			11	1	3				25	40			1	3	
Photobacterium sp. SS9	4	6	1		10	5	1		1	6	22	30			2	7	7
Strain DSJ4	2	5	2	4	3	3		2	1	15	9	32			1	9	13

Table 4. Levels of homology for the chromosomal DNAs of strain DSJ4 and *Photobacterium* reference strains

Strains	GC%	% Homology with DNA from:						
		ATCC 25915^T	ATCC 33539^T	JCM 8968^T	ATCC 25521^T	ATCC 11040^T	SS9	DSJ4
P. angustum ATCC 25915^T	39.6	100	25	30	34	24	24	21
P. damselae ATCC 33539^T	41.8	28	100	66	19	19	20	20
P. histaminum JCM 8968^T	41.8	22	65	100	17	16	29	16
P. leiognathi ATCC 25521^T	39.7	46	23	28	100	25	20	20
P. phosphoreum ATCC 11040^T	39.1	43	23	37	24	100	23	21
Photobacterium sp. SS9	41.5	27	23	24	17	19	100	78
Strain DSJ4	42.0	28	19	28	18	20	78	100

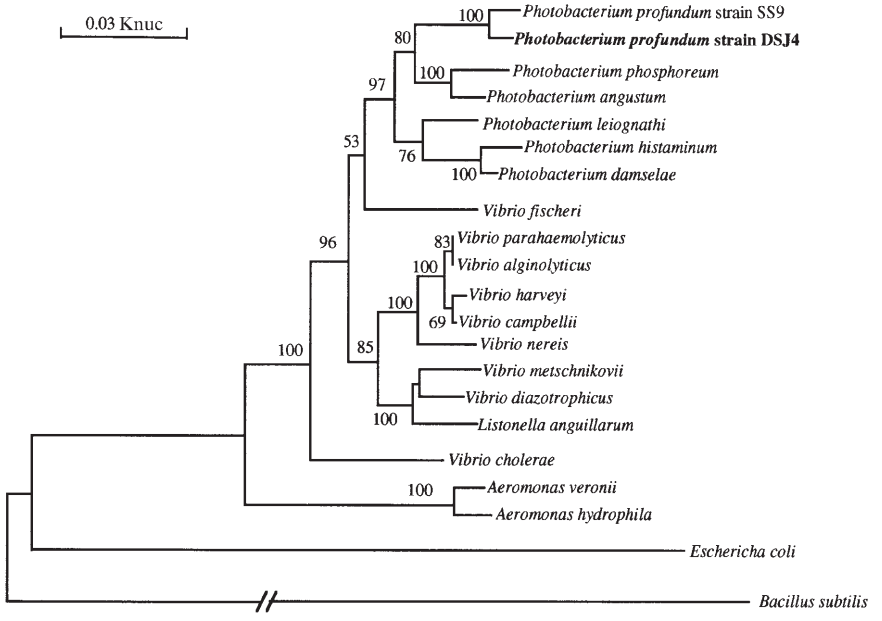

Fig. 3. Phylogenetic tree showing the relationship of amplified DSJ4 16S rDNA within the Proteobacteria γ-subgroup using the neighbor-joining method. The *scale* represents the average number of nucleotide substitutions per site. Bootstrap values are shown for frequencies above the threshold of 50%

Phylogenetic relationship based on 16S rDNA sequences

The results of phylogenetic analyses performed with 16S rDNA sequence information support the conclusions just described and further clarify the taxonomic and phylogenetic position of the new isolates among members of the genus *Photobacterium* and related genera. The phylogenetic tree shown in Fig. 3 shows that strains DSJ4 and SS9 form a cluster with all previously described members of the genus *Photobacterium*, but are located in a deeply branched, separate lineage within this cluster.

On the basis of phenotypic, genotypic, and phylogenetic data, it is logical to conclude that the isolates we studied are members of the genus *Photobacterium* and that the two deep-sea isolates constitute members of a new species within this genus. We propose the name *Photobacterium profundum* sp. nov. for strains DSJ4 and SS9 with strain DSJ4 as the type strain.

Description of *Photobacterium profundum* Nogi, Masui, and Kato sp. nov.

Photobacterium profundum Nogi, Masui, and Kato (sp. nov.) (pro.fun'dum L. adj.n *profundum* deep, living within the depth of the oceans). Cells are rod shaped; cell width ranges from 0.8 to 1.0 μm, and cell length ranges from 2 to 4 μm. Cells are Gram-negative and motile by means of a single unsheathed polar flagellum. Colonies on Marine Agar 2216 are entire, smooth, semitranslucent, nonluminescent, and ivory; they are 0.7–1.0 mm in diameter after 48 h of incubation at 10°C. The bacterium is halophilic

and psychrophilic. Best growth occurs at an NaCl concentration of ca. 3%. No growth occurs in the absence of NaCl. The optimal temperature for growth is between 8° and 12°C. No growth occurs at 0° or 20°C. It is a facultatively anaerobic chemoorganotroph having both respiratory and fermentative types of metabolism. Catalase and cytochrome oxidase positive. Acids are produced from D-glucose but gas is not produced. Nitrate is reduced to nitrite. The organism does not produce H_2S. The following compounds are utilized as sole carbon and energy sources: Tween 40, Tween 80, *N*-acetyl-D-galactosamine, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, D-trehalose, and glycerol. The following compounds are not utilized: adonitol, L-arabinose, lactose, D-raffinose, L-rhamnose, sucrose, and turanose. The G+C content of the DNA is about 42 mol%. The major isoprenoid quinone is Q-8. The dominant cellular fatty acids are 16:1, iso-16:0, 16:0, 18:1, and 20:5 ω3c (IPA).

The type strain is *Photobacterium profundum* sp. strain DSJ4, which has been deposited in the Japan Collection of Microorganisms as strain JCM10084.

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