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Colwellia piezophila sp. nov., a novel piezophilic species from deep-sea sediments of the Japan Trench

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Two strains of obligately piezophilic bacteria were isolated from sediment collected from the bottom surface of a small canyon on the seaward slope of the Japan Trench at a depth of 6278 m. The isolated strains, Y223G^T and Y251E, are closely affiliated with members of the genus *Colwellia* on the basis of 16S rRNA gene sequence analysis. The G+C contents of both strains were about 39 mol%. DNA–DNA hybridization values between these strains and *Colwellia* reference strains were significantly lower than those accepted as the phylogenetic definition of a species. The novel strains are Gram-negative, polarly flagellated and facultatively anaerobic. The optimal pressure for growth was 60 MPa at both 4 and 10 °C; the most rapid growth rate was observed at 10 °C and 60 MPa. No growth occurred at 15 °C under any pressure studied. The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are C16 : 0 and C16 : 1. Based on the taxonomic differences observed, the isolated strains appear to represent a novel obligately piezophilic *Colwellia* species. The name *Colwellia piezophila* sp. nov. (type strain Y223G^T=JCM 11831^T=ATCC BAA-637^T) is proposed.

Numerous deep-sea piezophilic bacterial strains have been isolated and characterized in an effort to understand the interaction between the deep-sea environment and its microbial inhabitants (Bartlett, 1999; Deming & Baross, 1993; Kato *et al*., 1995; Yayanos *et al*., 1979). Piezophilic bacteria, as defined by Yayanos (2001), are bacteria that have their maximum growth rate at high pressure over all permissible temperatures. All psychropiezophilic bacteria isolated to date are members of the *γ-Proteobacteria*, according to phylogenetic classifications based on 16S rRNA gene sequence information. Nogi *et al*. (2002) reported that cultivated psychropiezophilic deep-sea bacteria were affiliated with one of five genera within the *γ-Proteobacteria*: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* and *Psychromonas*.

In this paper, results of taxonomic studies on obligately piezophilic strains isolated from the deepest cold-seep environment in the Japan Trench are presented. Several lines of evidence indicate that two of these isolates, strains Y223G^T and Y251E, represent a novel species within the genus *Colwellia*. In the genus *Colwellia*, the only deep-sea piezophilic species reported is *Colwellia hadaliensis* strain BNL-1^T (Deming *et al*., 1988), although no public culture collection maintains *C. hadaliensis*. Species of the genus *Colwellia* are defined as facultative anaerobes and psychrophilic bacteria (Deming *et al*., 1988). Bowman *et al*. (1998) reported that *Colwellia* species produce docosahexaenoic acid (DHA; C22 : 6ω3). Psychropiezophilic *Shewanella* and *Photobacterium* strains produce eicosapentaenoic acid (EPA; C20 : 5ω3), *Moritella* strains produce DHA (Nogi & Kato, 1999; Kato & Nogi, 2001; Nogi *et al*., 1998a) and *Psychromonas kaikoae* produces both EPA and DHA (Nogi *et al*., 2002). Generally, psychropiezophilic bacteria produce either one or both of these long-chain polyunsaturated fatty acids (PUFAs). However, unlike other psychropiezophilic bacteria, our isolates did not produce either EPA or DHA in the membrane layer. Based on the taxonomic differences observed, the isolated strains appear to represent a novel obligately piezophilic *Colwellia* species, for which the name *Colwellia piezophila* sp. nov. is proposed.

Deep-sea sediment samples were collected by means of sterilized mud samplers (Ikemoto & Kyo, 1993) on the submersible *Shinkai 6500* from the bottom of a small deep-sea canyon on the Japan Trench (40° 2·8′ N 144° 16·6′ E) at a depth of 6278 m during cruise YK 01-06, dive #6K-621. The mud samples were taken by the submersible's

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFAs, polyunsaturated fatty acids.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Y223G^T is AB094412.

A 16S rRNA gene-based phylogenetic tree showing the relationship between *Colwellia piezophila* and piezophilic bacteria within the *γ-Proteobacteria* is available as supplementary material in IJSEM Online.

manipulator and put into the sample holder of the sterilized sampler. The samples were then carried to the sea surface without changing temperature, but with a change in pressure. A part of each sample was cultivated in marine broth 2216 (Difco Laboratories) at 4 °C and 60 MPa in a pressure vessel for about 2 weeks. Strains Y223G^T and Y251E were isolated using the low-melting agar method (Kato *et al.*, 1995). *Colwellia maris* JCM 10085^T (Yumoto *et al.*, 1998) and *Colwellia psychrerythraea* ATCC 27364^T (Deming *et al.*, 1988) were used as reference strains. These bacteria were maintained in marine broth 2216. *C. maris* was grown at 15 °C and atmospheric pressure, *C. psychrerythraea* was grown at 10 °C and atmospheric pressure and the isolated piezophilic strains were grown at 10 °C and 60 MPa. High-pressure cultivation utilized a liquid hydraulic system. Piezophilic bacteria were cultivated in plastic bags containing liquid medium in a pressure vessel (made of stainless steel SUS304). To supply oxygen to the cultures for the optimal growth pressure and temperature tests and the O/F test, fluorinert (FC-72; Sumitomo-3M) saturated with gas was added (20 % total volume). This culture method followed a previously reported procedure (Kato *et al.*, 1994; Yanagibayashi *et al.*, 1999).

Optimal growth pressure and temperature were determined by optical density. Cells were counted and cell form was confirmed microscopically in marine broth 2216 under aerobic conditions at each pressure and temperature tested.

Physiological tests were performed using a slight modification of the general procedures described by Barrow & Feltham (1993) and DeLong *et al.* (1997). All high-pressure physiological tests were performed in tandem with uninoculated blank controls according to the following procedure. Acid production from sugars was assessed using modified O/F medium (Hugh & Leifson, 1953) containing 0·5 × artificial sea water (1·5 % NaCl, 0·035 % KCl, 0·54 % $MgCl_2.6H_2O$, 0·27 % $MgSO_4.7H_2O$, 0·05 % $CaCl_2.2H_2O$), 0·05 % $NH_4H_2PO_4$, 0·005 % yeast extract, 0·1 % Na_2CO_3, 1 % sugar and 0·003 % bromothymol blue. The pH of the medium was adjusted to 7·1 at 20 °C. After capping, the tube was sealed with Parafilm and incubated at 60 MPa and 10 °C for several days (Nogi *et al.*, 2002). Physiological tests under high-pressure conditions to examine hydrogen sulfide production from thiosulfate and the production of indole, oxidase and catalase were performed according to previously described methods (Nogi & Kato, 1999). Gelatinase, protease and amylase activities were detected in ultrasonically treated cultured cells.

Cells of strain Y223G^T were Gram-negative rods, 2·0–3·0 μm long and 0·8–1·0 μm wide, motile by means of a single unsheathed polar flagellum. This strain was unable to grow at atmospheric pressure at 2–15 °C, although it grew well in pressure vessels under hydrostatic pressures of 10–80 MPa at 4 °C and 40–80 MPa at 10 °C. No growth occurred at 15 °C under any pressure examined. The most rapid growth rate (about 0·14 h^{-1}) was observed at 60 MPa and 10 °C (Fig. 1a), which compares well to rates observed for the closely related obligate piezophile *C. hadaliensis* (0·12 h^{-1} at 90 MPa and 10 °C; Deming *et al.*, 1988). The *C. psychrerythraea* reference strain was not able to grow under such high-pressure conditions (Fig. 1b) and the growth rate of *C. maris* showed greater pressure sensitivity.

Characteristics of the isolated piezophilic *Colwellia* strains and the reference strains are shown in Table 1. Strains Y223G^T and Y251E are facultatively anaerobic chemo-organotrophs, displaying both respiratory and fermentative types of metabolism. Other characteristics of Y223G^T and Y251E are as follows. Acid, but not gas, is produced from D-glucose, maltose and xylose. Catalase and cytochrome oxidase test results are positive and gelatin is hydrolysed. Nitrate is reduced to nitrite, but nitrite is not reduced. Test results for protease, chitinase, amylase, H_2S and indole production are negative. The following compounds are not utilized: L-arabinose, cellobiose, D-fructose, D-galactose, glycerol, *myo*-inositol, D-lactose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-sorbitol, sucrose and D-trehalose.

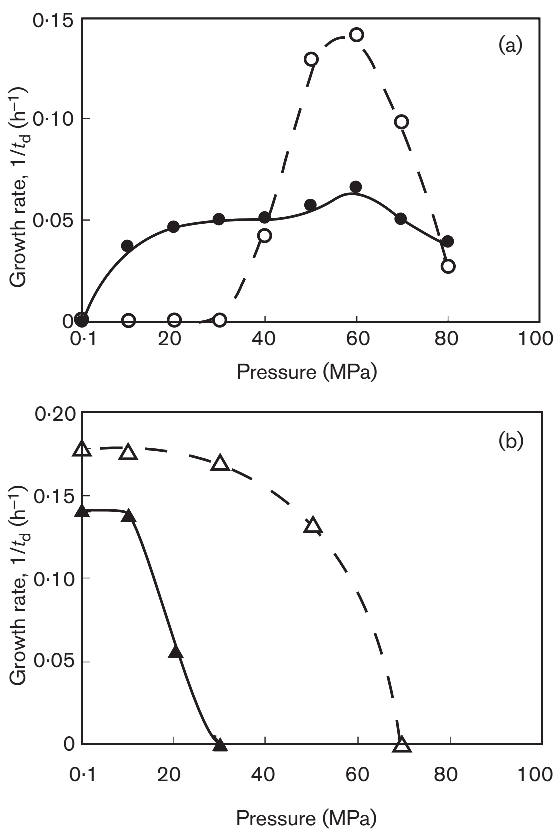

Fig. 1. (a) Pressure-dependent growth rate of *Colwellia piezophila* strain Y223G^T at 4 °C (●) and 10 °C (○). (b) Pressure-dependent growth rate of *C. psychrerythraea* at 10 °C (optimal temperature) (▲) and of *C. maris* at 15 °C (optimal temperature) (△). Growth rates were determined by optical density; t_d, doubling time (h).

Table 1. Phenotypic characteristics of isolates Y223G^T and Y251E, and related *Colwellia* strains

Species: 1, *C. piezophila* (Y223G^T and Y251E); 2, *C. maris*; 3. *C. psychrerythraea*. Data are from this study and Bowman *et al.* (1998). All species are Gram-negative rods that do not form spores and are motile by means of a polar flagellum. All strains are positive for catalase and oxidase reactions, hydrolysis of gelatin and nitrate reduction to nitrite. The major isoprenoid quinone type is Q-8. Acid is produced from D-glucose. All strains are negative for production of H_2S, indole, gas from carbohydrates and acid production from L-arabinose, D-fructose, D-galactose, glycerol, *myo*-inositol, D-lactose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-sorbitol, sucrose and D-trehalose. +, Positive; −, negative; ND, no data available.

Character	1	2	3
Optimum growth:			
Temperature (°C)	10	15	10
Pressure (MPa)	60	0·1	0·1
DNA G+C content (mol%)	38·5–39·1	36·9	40
Hydrolysis of:			
Casein	−	−	+
Chitin	−	−	+
Starch	−	+	+
Nitrite reduction to nitrogen	−	+	ND
Acid production from:			
Cellobiose	−	+	−
Maltose	+	−	+
Xylose	+	−	−

The G+C content of the DNA is 38·5–39·1 mol%. The major isoprenoid quinone is Q-8 (ubiquinone-8). The *C. maris* reference strain shares some physiological characteristics with the isolated strains, including the profile of carbohydrates utilized. However, unlike *C. maris*, the two Japan Trench strains do not hydrolyse starch, reduce nitrite or produce acid from cellobiose, and are unable to grow at atmospheric pressure. Although very few taxonomic characteristics of piezophilic *C. hadaliensis* are described in the literature (Deming *et al.*, 1988), the main characteristics in which it differs from the newly isolated strains are its ability to hydrolyse chitin and its DNA G+C content of 45·7 mol%.

To determine phylogenetic relationships, 16S rRNA gene sequences of strains Y223G^T and Y251E were determined by direct sequencing of PCR-amplified DNA according to a previously described method (Kato *et al.*, 1998). Nucleotide substitution rates (K_{nuc}; Kimura, 1980) were determined and a distance matrix tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the program CLUSTAL_W (Thompson *et al.*, 1994). Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the phylogenetic tree was evaluated by performing bootstrap analysis with 1000 bootstrapped trials. The results of phylogenetic analyses based on 16S rRNA gene sequence information are shown in Fig. 2 (see also the phylogenetic tree available as supplementary material in IJSEM Online). Strains Y223G^T and Y251E fall into the genus *Colwellia* and are closely related to the psychrophilic strain *C. maris*. For analysis of relatedness, DNA–DNA hybridization was carried out at 40 °C for 4 h and measured fluorometrically using the method of Ezaki *et al.* (1989). A high level of DNA–DNA relatedness (98–100 %) was found between strains Y223G^T and Y251E. The relatedness between Y223G^T or Y251E and the *C. maris* reference strain was less than 49 %, as indicated by the hybridization values obtained. This is significantly lower than that accepted as the phylogenetic definition of a species (Wayne *et al.*, 1987). This and other results shown in Table 1 and Fig. 2 suggest that strains Y223G^T and Y251E represent a novel *Colwellia* species.

The whole-cell fatty acid compositions of strains Y223G^T and Y251E and the reference strains are shown in Table 2. The major fatty acids in strains Y223G^T and Y251E were C16 : 0 (hexadecanoic acid) and C16 : 1 (hexadecenoic acid). Only a low level of similarity was observed between this fatty acid profile and those of the type strains of *C. maris* and *C. psychrerythraea*. For example, the predominant components in the fatty acid profile of the *C. maris* type strain differed from those in the profile of strains Y223G^T and Y251E, which contained substantial amounts of C14 : 0 (tetradecanoic acid), C17 : 0 (heptadecanoic acid) and C17 : 1 (heptadecenoic acid). The type strain of *C. psychrerythraea* had low levels of the long-chain PUFAs EPA and DHA, unlike strains Y223G^T and Y251E, which did not contain these fatty acids. Psychropiezophilic *Shewanella* and *Photobacterium* strains produce EPA (Nogi *et al.*, 1998b, c), *Moritella* strains produce DHA (Nogi & Kato, 1999) and *P. kaikoae* produces both EPA

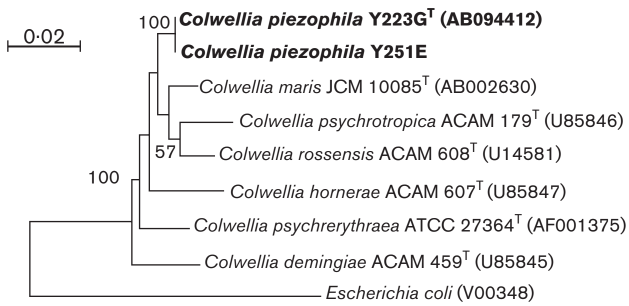

Fig. 2. Phylogenetic tree showing the relationships of strains Y223G^T and Y251E within the genus *Colwellia* constructed by the neighbour-joining method and based on 16S rRNA gene sequences. Bar, 0·02 nucleotide substitutions per site. Bootstrap values were calculated from multiple resamplings of the sequence dataset, which are the basis for multiple tree topologies. A fuller phylogenetic tree showing the position of the isolates in relation to psychropiezophilic strains is available as supplementary data in IJSEM Online.

Table 2. Whole-cell fatty acid composition (%) of piezophilic *Colwellia* isolates, other *Colwellia* species and piezophilic species of related genera

Strains: 1, *Colwellia piezophila* Y223G^T and Y251E; 2, *Colwellia maris* JCM 10085^T (data from this study); 3, *Colwellia psychrerythraea* DSM 8813^T (data from Bowman *et al.*, 1998); 4, *Moritella japonica* JCM 10249^T; 5, *Photobacterium profundum* JCM 10084^T; 6, *Psychromonas kaikoae* JCM 11054^T; 7, *Shewanella benthica* ATCC 43992^T (data for columns 4–7 from Nogi *et al.*, 2002).

Fatty acid	1	2	3	4	5	6	7
12 : 0	1–2	2			2	1	2
14 : 0	9		5·1–7·8	18	3	6	14
15 : 0	3	4	1·7–11·0	1	1	1	
16 : 0	31–33	25	26·8–33·2	21	9	15	14
17 : 0		3	0·0–1·3				
18 : 0			0·1–2·4		1		
iso-13 : 0					2		5
iso-14 : 0					4		
iso-15 : 0					2		11
iso-16 : 0					15		
14 : 1	2	2	5·1–7·3	2	3	10	
15 : 1	3	6					
16 : 1	48–50	51	37·6–45·1	50	31	54	31
17 : 1		4	0·0–2·2				
18 : 1			0·3–2·1	2	9	2	2
20 : 5 (EPA)			0·0–1·5			2	16
22 : 6 (DHA)			5·5–8·0	6	13	2	
3-OH 12 : 0	0–1	3			5	2	1
3-OH iso-13 : 0							5
3-OH 14 : 0						4	

and DHA (Nogi *et al.*, 2002). Generally, psychropiezophilic bacteria are believed to produce EPA and/or DHA. However, Allen *et al.* (1999) reported that monounsaturated fatty acids, but not PUFAs, are required for growth of the piezophilic bacterium *Photobacterium profundum* SS9, based on analysis of pressure-sensitive mutants. In their mutation experiment, C18 : 1 fatty acid was necessary for growth under low-temperature and/or high-pressure conditions. However, our strains did not contain any monounsaturated fatty acids longer than C16 : 1. Therefore, our isolates differ from previously known psychropiezophilic bacteria in that most of the unsaturated fatty acids of these isolates have been identified as C16 : 1 (Table 2).

Several psychropiezophilic bacterial strains from the cold deep sea have been isolated and characterized (MacDonell & Colwell, 1985; Nogi & Kato, 1999; Nogi *et al.*, 1998a, b, c, 2002); each is affiliated with one of five genera within the *γ-Proteobacteria*: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* and *Psychromonas* (DeLong *et al.*, 1997; Nogi *et al.*, 2002). The first obligately piezophilic bacterium, strain MT41 isolated from the Mariana Trench, was later shown to be a member of the genus *Colwellia* (Yayanos *et al.*, 1981; DeLong *et al.*, 1997). Since then, four obligately piezophilic bacterial strains have been isolated: *C. hadaliensis* BNL-1^T (Deming *et al.*, 1988), *Shewanella benthica* DB21MT-2, *Moritella yayanosii* DB21MT-5^T (Nogi & Kato, 1999) and *P. kaikoae* JCM 11054^T (Nogi *et al.*, 2002). A novel obligately piezophilic species within *Colwellia*, based on the results of phylogenetic analysis of 16S rRNA gene sequences and several other taxonomic properties described in this paper, is therefore proposed.

On the basis of the phenotypic, genotypic and phylogenetic data, it is logical to conclude that our isolates are members of the genus *Colwellia* and that these deep-sea isolates represent a novel species within this genus. The name *Colwellia piezophila* sp. nov. is proposed, with strain Y223G^T (=JCM 11831^T=ATCC BAA-637^T) as the type strain.

Description of *Colwellia piezophila* sp. nov.

Colwellia piezophila (piez.o.phi′la. Gr. v. *piezo* to press; Gr. adj. *philos* loving; N.L. fem. adj. *piezophila* loving pressure).

Cells are Gram-negative rods, 2·0–4·0 × 0·8–1·0 μm, motile by means of a single unsheathed polar flagellum. Halophilic, psychrophilic and piezophilic. Optimal growth occurs at an NaCl concentration of about 3 %. No growth occurs in the absence of NaCl. The optimal temperature and pressure for growth are 10 °C and 60 MPa, respectively. No growth occurs at atmospheric pressure and 2–15 °C or at 15 °C under any pressure. Facultatively anaerobic chemoorganotroph, having both respiratory and fermentative types of metabolism. Catalase and cytochrome oxidase test results are positive, gelatin is hydrolysed, nitrate is reduced to nitrite, but nitrite is not reduced. Amylase, protease, H_2S production and indole production are negative. The DNA G+C content of the type strain, Y223G^T (=JCM 11831^T=ATCC BAA-637^T), is about 39·1 mol%. The major isoprenoid quinone is Q-8. The predominant cellular fatty acids are C16 : 0 and C16 : 1. Other characteristics are shown in Table 1.

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