Colwellia marinimaniacae sp. nov., a hyperpiezophilic species isolated from an amphipod within the Challenger Deep, Mariana Trench

Masataka Kusube, Than S. Kyaw, Kumiko Tanikawa, Roger A. Chastain, Kevin M. Hardy, James Cameron and Douglas H. Bartlett.*

Abstract

An obligately piezophilic strain was isolated from an amphipod crustacean obtained in the Challenger Deep region of the Mariana Trench during the DEEPSEA CHALLENGE expedition. The strain, MTCD1T, grew at extremely high hydrostatic pressures, with a growth range of 80–140 MPa (optimum, 120 MPa) at 6 °C. Phylogenetic analyses based on the 16S rRNA gene sequence indicate that it is closely affiliated with the genus Colwellia. Comparative 16S rRNA gene sequence analyses revealed 95.7, 95.5 and 95.2 % similarity to Colwellia maris ABE-1T, Colwellia piezophila Y233G1 and Colwellia psychrerythraea ATCC 27364T, respectively. The major cellular fatty acids were C16:1, C16:0 and C22:6 (docosahexaenoic acid), and the sole isoprenoid quinone produced was ubiquinone-8. DNA G+C content was 48.6 mol%. The strain was positive for oxidase and catalase activities. Based on the results from this study, strain MTCD1T is a novel Gram-negative species of the genus Colwellia, and the name Colwellia marinimaniacae sp. nov. (type strain MTCD1T=ATCC TSD-5T=JCM 30270T) is proposed. It is the most piezophilic organism yet described.

Members of the genus Colwellia (class Gammaproteobacteria, order Alteromonadales) are rod-shaped, Gram-negative, facultatively anaerobic marine microbes capable of motility via a polar or subpolar flagellum and anaerobic respiration using nitrate as a terminal electron acceptor [1]. Since its formal description in 1988 [2], 15 species within the genus have been described. Most are noteworthy for their growth preferences at low temperatures (possessing psychrophilic or psychrotolerant phenotypes) and in some cases at high hydrostatic pressures as well (displaying a piezophilic phenotype). Six of the psychrophilic species include Colwellia demingiae, C. hornerae, C. maris, C. psychrerythraea (strains ATCC 27364T, GABI4E and ND2E), C. rossensis and C. psychrotropica [2–5]. The six psychrotolerant species include Colwellia aestuarii, C. polaris, C. asteriadis, C. chukchienis, C. aquamaris and C. arctica [6–11]. Thus far, one mesophilic species, designated Colwellia meonggi, has been isolated [12].

Piezophiles are micro-organisms whose growth rates are optimal at pressures above atmospheric pressure but less than 60 MPa hydrostatic pressure, and hyperpiezophiles possess superior growth rates above 60 MPa [13]. Since the formation of the genus Colwellia, it has been associated with extremophiles capable of high-pressure growth. It was originally proposed by Deming et al. [2] after characterizing a hyperpiezophilic psychrophile, designated Colwellia hadaliensis BNL-1T, obtained from seawater collected at a depth of ~7400 m in the Puerto Rico Trench. The hyperpiezophilic psychrophilic strain MT41 was isolated from an amphipod crustacean collected at a depth estimated to be ~10 500 m in the Mariana Trench [14] and was later assigned to the genus Colwellia [15]. This strain was the first obligate piezophile obtained and is unable to grow at pressures less than 35 MPa [16]. At atmospheric pressure, MT41 cells eventually lyse [17]. Colwellia piezophila is another hyperpiezophilic psychrophile and obligate piezophile within the genus. It was isolated from sediments recovered from a depth of ~6300 m in the Japan Trench [18]. Thus, psychrophilic and obligately piezophilic Colwellia members have been obtained from animals, seawater and sediments collected from hadal depths within ocean trenches in both the Pacific and Atlantic oceans. The effect

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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MTCD1T is AB971664.

Two supplementary figures are available with the online Supplementary Material.
of increased hydrostatic pressure on one other member of the genus *Colwellia* has been characterized. *C. psychrerythraea* ATCC 27364ᵀ isolated from Arctic sea ice at −300 m depth shows optimum growth at 0.1–10 MPa and 8 °C, but growth rate rapidly declines to undetectable levels at −30 MPa [18–20].

Another noteworthy characteristic of many *Colwellia* species is the production of long-chain omega-3 polyunsaturated fatty acids (PUFAs) [3, 21]. They can contain docosahexaenoic acid (DHA; C22:6ω3) and eicosapentaenoic acid (EPA; C20:5ω3) in their membranes. While long-chain PUFAs are present in many piezophiles, including strain MT1 [21–25], they do not appear to exist in *C. piezophila* [18] and thus may not be a requirement for either psychrophyly or piezophily in this genus.

During the DEEPSEA CHALLENGE expedition (www.deepseachallenge.com/; [26]), we isolated a hyperpiezophilic psychrophile from the Challenger Deep with the Mariana Trench. The Challenger Deep includes the deepest location on Earth, with depths reaching ~11 092 m [27]. The strain was obtained from a decaying *Hirondella gigas* amphipod, collected in a 30 litre Niskin bottle baited with skipjack tuna, which was deployed on the seafloor via an autonomous lander system [28]. The location was the middle portion of the Challenger Deep (11.36902° N 142.43294° E), and the depth, as recorded by a RBR DR-1050 pressure sensor and derived using the UNESCO pressure to depth equation [29], was 10 918 m. Upon retrieval of the lander on the Indonesian vessel Barakuda, the temperature of the seawater and associated amphipods was 6.5 °C. Amphipods and seawater were emptied into a sterile teflon bag placed inside a chilled tub. Subsequently, amphipods and 0.22-µm-filter-sterilized trench seawater were quickly transferred into polyethylene bags under low light conditions, heat sealed and pressurized to 110 MPa using a liquid hydraulic hand pump. The pressure vessel was maintained pressurized and at temperatures equal to or less than 5 °C until the sample was used for microbial enrichment several weeks later at Scripps Institution of Oceanography.

*C. marinimaniae* strain MTCD1ᵀ was isolated from this material using strategies similar to those previously employed for psychrophilic and piezophilic deep-sea bacteria [14, 30]. Other piezophiles have previously been isolated from the Mariana Trench, including *Dermacoccus abyssi* MT1.1ᵀ, *Moritella yayanosii* JCM 10263ᵀ, *Shewanella benthica* DB21MT-2 and, as noted above, *Colwellia* sp. MT41 [14, 23, 31–33]; however, a hyperpiezophile with an optimum growth pressure above 110 MPa has not previously been recovered from any location. The strain name ‘MTCD1’ originates in isolation place, i.e. Mariana Trench Challenger Deep and sample number one. Based upon its 16S rRNA gene sequence, it is phylogenetically distinct from other taxonomically characterized species in the genus *Colwellia* but is closely related to *Colwellia* strain MT41. The objective of the present study was to establish the taxonomic position of strain MTCD1ᵀ using polyphasic taxonomic approaches. A single MTCD1ᵀ strain was used for this study with the reference strain *C. psychrerythraea* ATCC 27364ᵀ (AF001375).

Strain MTCD1ᵀ was isolated from the obtained amphipod-associated seawater following dilution of this material into a solid medium and isolation of one of the colonies. Culturing was routinely performed in HEPES-buffered marine broth 2216 medium. It contained 37.4 g marine broth 2216 (Difco) l⁻¹. After autoclaving, 0.2-µm-filter-sterilized HEPES buffer (pH 7.5) was added aseptically to a final concentration of 100 mM, and for solid medium, autoclaved 12 % gelatin dissolved in artificial seawater (Sigma) and maintained at 20 °C was included to a final concentration of 4 %. The cultivation temperature was 6 °C, unless indicated otherwise. Colony growth and morphology was examined in the solid medium after incubation for a couple of weeks at 6 °C under high hydrostatic pressure in sealed plastic bulbs. Gram staining was performed according to Dussault [34] after fixation under 100 MPa. Cell morphology was examined using scanning electron microscopy (Fig. 1). Motility was checked using semi-solid gelatin medium containing 1 % agar and 0.5 % gelatin [32] and by microscopic observation.

Bacteria were grown in the marine broth 2216 medium to determine their growth characteristics. The pressure range for growth was assayed from 0.1 to 150 MPa. The influence of temperature was determined at 4.0, 6.0, 8.0 and 10.0 °C. High hydrostatic pressure cultivation was accomplished using two different systems. Initially, cultivation was performed in polyethylene Samco bulbs (ThermoFisher Scientific) placed within a 2 litre internal volume thermally jacketed, custom-designed batch incubator manufactured by Parker Autoclave Engineers. Pressurization was performed using an air compressor connected to a hydraulic pumping system. Later, high-pressure incubations utilized a

![Fig. 1. Scanning electron micrograph of *C. marinimaniae* sp. nov. MTCD1ᵀ. Bar, 1 µm.](image-url)
PV200 vessel (Syn) with pressurization based on a high-pressure pump (AP200, Syn). Cell numbers and bacterial growth rates were determined using 4′,6′-diamidino-2-phenylindole VectaShield (Vector Laboratories) in conjunction with an Olympus BX51 fluorescent microscope equipped with UV and blue light filter cubes. Strain MTCD1T displayed an optimum growth rate of 0.08 h⁻¹ at 120 MPa and 6°C (Figs 2 and S1, available in the online Supplementary Material). Moreover, this strain was able to grow at 140 MPa and 6°C (0.07 h⁻¹), 120 MPa and 4°C (0.06 h⁻¹) and 120 MPa and 8°C (0.05 h⁻¹) but could not grow at atmospheric pressure or under high pressure at 10°C (Figs 2 and S2). These growth rate results are similar to those of other obligate piezophiles, i.e. C. hadalensis BNL-1T [2], C. piezophila Y223G T [18] and Psychromonas kaikoae JCM 11054T [35]. However, MTCD1T possesses greatest similarity to Colwellia species strain MT41. The two strains were both isolated from hadal regions of the Mariana Trench and represent the two most piezophilic strains of bacteria or archaea isolated to date. Strain MTCD1T is slightly more psychrophilic and piezophilic than strain MT41 [16]. It has a 6°C temperature optimum and a 120 MPa pressure optimum versus a 8°C temperature optimum and a 110 MPa pressure optimum, respectively.

Acid formation from sugar was tested according to Nogi et al. [18] using media supplemented with 0.003 mg bromothymol blue l⁻¹ as an indicator. Tests for oxidase were performed using API 20NE (Sysmex). Nitrate and nitrite reduction assays followed the general procedures described by Nogi et al. [18]. Hydrogen sulfide production from thiosulfate and indole production from tryptophan were performed by cultivation in sulfide indole motility (SIM) medium [32] at 6°C, 120 MPa. The activities of two proteases (caseinase and gelatinase), along with chitinase, amylase and catalase, were assessed using modified general plate methods [36] with 1% casein, 4% gelatin, 0.05% ethylene glycol chitin and 1% starch as substrates, respectively. The incubation temperature and pressure for the tests were 6°C and 120 MPa, and the incubation times were 4 weeks. H₂S formation was determined by black sulfide precipitation in medium containing 0.2 g sodium thiosulfate l⁻¹ [37]. The results of these tests are included in the species description, and the differences between the MTCD1T strain and related species are highlighted in Table 1. C. psychrerythraea ATCC 27364T was used as a comparison for the hydrolysis and acid production tests. Cells of MTCD1T are Gram-negative, motile, with less than 50 mol% G+C content. The oxidase and catalase activities are positive, and chitinase, amylase, nitrate reduction, nitrite reduction and indole production are negative. Except for the lack of nitrate reduction, these results are all shared with one or more of the Colwellia species C. marinimaniae, C. piezophila, C. maris and C. psychrerythraea. The lack of observed nitrate or nitrite reduction is curious as the genes for dissimilatory nitrate reduction and nitrite reduction are present in the genome of MTCD1T (Kyaw et al., unpublished results).

Cellular fatty acids were extracted from the middle to end portion of the logarithmic phase of growth [38, 39]. Methyl esterification of the cellular fatty acids and purification of the methylated fatty acids were conducted using the fatty acid methylation kit (06482-04, Nacalai Tesque) and the methylated fatty acid purification kit (06483-09, Nacalai Tesque) [40]. Analysis of cellular fatty acids was performed using GC/MS-QP2010 (Shimadzu) with Inert Cap WAX column (GL Science). Cellular fatty acid components were analysed with cells grown in marine broth 2216 for 2 weeks. The major fatty acids of strain MTCD1T were C₁₆:₀ (47%), C₁₆:₁ (18%), C₂₂:₆ (DHA, 17%), C₁₄:₁ (8%), C₁₈:₁ (3%), C₁₈:₀ (3%) and C₂₀:₅ (EPA, 2%). A comparison of the fatty acid profiles of various Colwellia species is provided in Table 2. The fatty acid profile of strain MTCD1T was in good agreement with strain MT41 and C. psychrerythraea ATCC 27364T. In addition to the production of large amounts of C₁₆:₀ and C₁₈:₁ω₇c, all three indicated Colwellia strains produce distinctive levels of C₁₈:₀ and C₁₈:₁ω₇c and also produced the PUFA, DHA. Indeed, strains MTCD1T

Fig. 2. Pressure-dependent growth rate of C. marinimaniae sp. nov. MTCD1T at 6°C (open circle) and 10°C (filled circle) compared to that previously determined for C. hadalensis BNL-1T [2] at 10°C (open triangle), C. piezophila Y223G T [18] at 10°C (filled triangle) and Psychromonas kaikoae JCM 11054T [35] at 10°C (open square). The growth rate refers to the inverse of the doubling time (t₀). Cell numbers were determined by 4′,6′-diamidino-2-phenylindole staining and fluorescence microscopy.
Isoprenoid quinones were extracted from 100 mg of freeze-dried cells with 100 ml chloroform/methanol (2:1, v/v) overnight at room temperature. The extracted solution was filtered and concentrated. This solution was separated on a TLC plate (PLC Silicagel 60 F254, 2 mm; Merck) using benzene as the solvent and recovered from the TLC plate with acetone. The obtained isoprenoid quinones were analysed by HPLC equipped with a SimPack VP-ODS C18 column (4.6×250 mm) (Nacalai Tesque). DNA G+C content was determined using the QIAquick Gel Extraction kit (Qiagen). Sequencing of the 16S rRNA gene, including the hypervariable region [4], was performed by Operon Technologies. A phylogenetic tree was reconstructed using the neighbour-joining method (Fig. 3), which was evaluated by bootstrap sampling and expressed as percentages of 1000 replicates. The strain MTCD1T 16S rRNA gene sequence is phylogenetically closely related to species in the genus Colwellia: strain MT41 (99.9%), C. maris ABE-1T (95.7%), C. piezophila Y233G T (95.5%) and C. psychrerythraea ATCC 27364T (95.2%). The trees (Fig. 3) also supported the conclusion that strain MTCD1T belongs within the genus Colwellia and that it is clearly a unique species [18, 8]. The MTCD1T and MT41 16S rRNA gene sequences are virtually identical, with 1 bp difference.
Table 2. Cellular fatty acid composition of strain MTCD1T along with other type strains in the genus *Colwellia* as well as other piezophiles

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<td>6–8</td>
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Phenotypic, chemotaxonomic and phylogenetic data based on 16S rRNA gene sequence comparison support the placement of strain MTCD1T as a novel species in the genus *Colwellia*. We propose strain MTCD1T to be the type strain for the new species *C. marinimaniae* sp. nov., which consists of two members, MTCD1T and MT41.

**DESCRIPTION OF COLWELLIA MARINIMANIAE SP. NOV.**

*Colwellia marinimaniae* (ma.ri.ni.ma.ni.ae). N.L. gen. n. *marinimaniae* named in honour of Marine Mania, a high school science group, located on the island of Guam.) In recent years this organization has been active in outreach programmes related to the Mariana Trench.

Cells are Gram-negative, motile, anaerobic chemoorganotroph rods (0.4–0.5×0.8–1.2 μm) during exponential growth phase. Colonies in Marine Medium 2216 gelatin bulbs are light beige with diameters of 0.5–1.0 mm after incubation for 2 weeks. Halophilic, psychrophilic and piezophilic. Cells grow in liquid marine broth 2216 medium over a pressure range of 80–140 MPa (optimum, 120 MPa) and 6.0 °C. It is the most piezophilic microbe described thus far. The oxidase and catalase activities are positive, and chitinase, amylase, nitrate reduction, nitrite reduction and indole production are negative. H₂S is not produced from sodium thiosulfate. Gelatin, but not casein, is hydrolysed. The following substrates are utilized as single carbon sources: maltose, D-xylose, cellobiose, glycerol and mannose. Acid is not produced from maltose, D-xylose, cellobiose, glycerol and mannose. The major cellular fatty acids are C₁₆:1ω₁, C₁₆:0 and DHA, and the major isoprenoid quinone is ubiquinone-8. The principal data used to distinguish strain MTCD1T from species in the
The type strain is MTCD1<sup>T</sup> (＝ATCC TSD-5<sup>T</sup>＝JCM 30270<sup>T</sup>). It was isolated from the Challenger Deep region.
of the Mariana Trench. The DNA G+C content of the type strain is 48.6 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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