Assignment of *Vibrio* sp. strain ABE-1 to *Colwellia maris* sp. nov., a new psychrophilic bacterium

Isao Yumoto,¹ Kosei Kawasaki,¹ Hideaki Iwata,¹,² Hideatoshi Matsuyama² and Hidetoshi Okuyama¹,³

Author for correspondence: Isao Yumoto. Tel: +81 11 8578925. Fax: +81 11 8578900. e-mail: yumoto@hniri.go.jp

¹ Bioscience and Chemistry Division, Hokkaido National Industrial Research Institute, Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan

² Department of Bioscience and Technology, School of Engineering, Hokkaido Tokai University, Minaminosawa, Minami-ku, Sapporo 005-8601, Japan

³ Laboratory of Environmental Molecular Biology, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan

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**INTRODUCTION**

There have been many reports about micro-organisms that can grow in extreme environments such as low and high temperatures, high pressure, alkaline and acidic conditions, and high chemical concentrations (Horikoshi & Grant, 1991). Even though the temperature is below 10 °C in almost 80% of the Earth, little is known about the taxonomic diversity and physiology of micro-organisms which have adapted to the cold. Morita (1975) defined the psychrophile as having an optimum temperature for growth of about ≤ 15 °C and a maximum temperature for growth of ≤ 20 °C, and the psychrotroph as a cold-tolerant organism, with a maximum growth temperature above 20 °C. Compared with mesophiles, there are not many examples of isolation of psychrophiles. However, several strains have been identified at the species level (Bowman et al., 1997a, b; D’aoust & Kunshner, 1972; Deming et al., 1988; Gounot, 1976; Irgens et al., 1996; Morita & Haight, 1964; Pacha, 1968).

A psychrophilic bacterium, *Vibrio* sp. ABE-1T, has been isolated previously (Takada et al., 1979). The micro-organism exhibited optimum and maximum temperatures for growth of 15 °C and below 24 °C, respectively. The cellular fatty acids concerned with cold adaptation (Okuyama et al., 1990, 1991), bioenergetic properties as a marine bacterium (Takada et al., 1981, 1989a, 1991), and enzymology as a psychrophile (Ishii et al., 1987, 1993) of the strain have been studied extensively. However, its taxonomic position at the species level remains to be determined.

In this study, phenotypic and chemotaxonomic characteristics and the phylogenetic position of strain ABE-1T have been examined; results showed that the strain should be classified as a new species.
**METHODS**

**Bacterial strains and cultivation.** The strain examined was *Vibrio* sp. ABE-1<sup>1</sup> (Takada *et al.*, 1979). It was isolated from seawater which was obtained from the Abashiri coast (44°3' N, 144°16' E), off the Okhotsuku Sea, in Hokkaido, Japan, in the season when drifting ice from Siberia flowed into the region. The organism was cultured aerobically until the late exponential phase of growth at 15 °C in PYSE medium (pH 7.5), unless otherwise stated. The PYSE medium (pH 7.5) contained (per litre distilled water) 30 g NaCl; 0.7 g KCl; 5.3 g MgSO<sub>4</sub>·7H<sub>2</sub>O; 1.3 g CaSO<sub>4</sub>·2H<sub>2</sub>O; and 10.8 g MgCl<sub>2</sub>·6H<sub>2</sub>O. *Colwellia psychrerythraea* ATCC 27364<sup>1</sup> (type strain) was used as a comparison for the test of DNA–DNA hybridization. The strain was grown at 15 °C on marine agar or broth 2216 (Difco).

**Electron microscopy.** Cells which were grown on PYSE agar medium were suspended in physiological saline. A small drop of the suspension was placed on a carbon-coated copper grid and negatively stained with 1% phosphotungstic acid for observation with a transmission electron microscope (Hitachi H-800).

**Analysis of isoprenoid quinones.** Isoprenoid quinones were extracted by treating 500 mg freeze-dried cells with 150 ml chloroform/methanol (2:1, v/v) for 2 h in a reciprocal shaker (120 strokes per min) at room temperature. The extracted solution was concentrated and transferred by acetone. The resulting solution was concentrated, separated by TLC using n-hexane/dimethyl ether (85:15, v/v) as the solvent, and recovered from the TLC plate using acetone. The obtained isoprenoid quinones were analysed by HPLC equipped with a Novapak C18 column (Waters) at room temperature. The HPLC system consisted of a solvent delivery pump (model CCPM-11; Tosoh) and a spectrophotometer detector (model UV-8020; Tosoh) set at 275 nm.

**Analysis of cellular fatty acids.** Whole cell fatty acids were extracted from 100 mg freeze-dried cells, which were cultivated on PYSE medium, esterified by acid methanolysis and analysed by GC equipped with a flame-ignition detector (model GC-7A; Shimadzu) and a 25 m BPX70 column (SGE) with an oven temperature of 170 °C. Fatty acids were identified by comparing them with fatty acid methyl ester standards (Supelco) and by GC/MS (model INCOS 50; Finnigan mat) connected to GLC (model 3400; Varian).

**DNA base composition and DNA–DNA hybridization.** DNA was prepared from bacterial cells by the method of Marmur (1961). The DNA G+C content was determined by the method of Tameoka & Komagata (1984). The obtained DNA was digested with nuclease P1 (Yamasu Shoyu). The resulting nucleotides were separated by HPLC. The HPLC system was as described above. An equimolar mixture of four deoxyribonucleotides (Yamasu Shoyu) was used as the standard.

**Levels of DNA relatedness were determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microplates.**

**Amplification of 16S rRNA gene and sequencing.** The 16S rRNA gene was amplified by PCR. The sequences of the primers used for amplification were 5′ AGAGTTTGAT-CCTGCT 3′ and 5′ AAGAGTTGATCAGCCGCA 3′, corresponding to positions 8–24 and 1528–1544, respectively, in the 16S RNA sequence of *Escherichia coli* (Brosius *et al.*, 1978). The 1.5 kb PCR product was directly sequenced by the dideoxynucleotide chain-termination method with a DNA sequencer (model 377; Applied Biosystems). Multiple alignments of the sequence were performed, nucleotide substitution rates (**K**<sub>n</sub>) value were calculated, and a neighbour-joining phylogenetic tree (Kimura, 1980; Saitou & Nei, 1987) was constructed using the CLUSTAL W program (Thompson *et al.*, 1994). The similarity values of the sequences were calculated using the GENETYX computer program (Software Development).

**Nucleotide sequence accession numbers.** The DDBJ/EMBL/GenBank accession numbers for the sequences used as reference sequences are as follows: *Colwellia*-like bacterium 551-W(gv)1 (Gosink & Stakey, 1995), U14581; *C. psychrerythraea* (DeLong *et al.*, 1993), L10939; small subunit rRNA gene cloned from macroaggregate of the sea (AGG53) (DeLong *et al.*, 1993), L10950; *Pseudoalteromonas haloplankis* subsp. haloplankis ATCC 14393<sup>1</sup>, X67024; *Shewanella hansenii* CIP 103207<sup>1</sup>, X82132; *Photobacterium angustum* ATCC 25915<sup>1</sup>, X74685; *Vibrio cholerae* ATCC 14035<sup>1</sup>, X74695; *Alteromonas macleodi* IAM 12920<sup>1</sup>, X82145; *Aeromonas hydrophila* ATCC 35654, X74676; *Escherichia coli* (Carbon *et al.*, 1979), J01859; *Moritella marinus* NCIMB 1144<sup>1</sup>, X82142; *Marinomonas vaga* ATCC 27119, X67025.

**RESULTS**

**Morphology**

When the bacterium was grown at 15 °C in PYSE medium, the cells appeared as single motile curved rods of 0·6–0·8 by 2·0–4·0 μm in size. Flagella-staining light microscopic observation and transmission electron micrograph (Fig. 1) revealed a single polar flagellum. Spore formation was absent and Gram-staining was negative.

**Phenotypic characteristics**

Strain ABE-1<sup>1</sup> exhibited the following physiological and biochemical characteristics. Oxidase and catalase activities were positive. Acids were produced from D-glucose under aerobic conditions; no acids, however,
were produced under anaerobic conditions. No acids were produced from L-arabinose, D-fuctose, maltose, D-mannose, melibiose and sucrose under either aerobic or anaerobic conditions. The strain did not grow in the absence of NaCl in the culture medium. It grew in media supplemented with 3 and 4% NaCl, but not in media with salinity higher than 6.5% NaCl. Susceptibility to the vibriostatic compound O/129 (10 and 150 μg) was also detected. Growth occurred at 0-22 °C, but not at temperatures higher than 25 °C. The strain was negative for methyl red, Voges–Proskauer and indole production, but did reduce nitrate to nitrite. It hydrolysed gelatin, DNA and Tweens 20, 40, 60 and 80, but not casein, chitin, starch or alginic acid. The strain utilized D-glucose as a sole carbon and energy source for growth, but not D-mannose, raffinose, D-xylose, L-arabinose, D-fructose, glycerol, lactose, maltose, melibiose or sucrose.

Chemotaxonomic characteristics

The isoprenoid quinones isolated from strain ABE-1T using TLC were analysed by HPLC. Analysis revealed ubiquinone-8 (Q-8) was the predominant isoprenoid quinone in the strain. GC analysis of the methyl ester derivatives of the fatty acids of the strain revealed that the major components were C₁₆:₀ (18%), C₁₆:₁ 9t (20%) and C₁₈:₁ 9c (20%). Smaller, but substantial amounts of C₁₀:₀ (6%), C₁₇:₀ (5%) and C₁₈:₁ 11c (6%) were also detected.

16S rRNA sequence analysis

The almost complete 16S rRNA sequence of strain ABE-1T, consisting of 1514 nucleotides, showed 92.9% similarity to the 16S rRNA sequence of C. psychrerythraea, whereas its similarity to the sequences of Pseudoalteromonas, Shewanella, Photobacterium, Vibrio, Moritella, Alteromonas, Escherichia, Pasteurella and Aeromonas species was less than 90%. The strain showed high similarity (97.8%) with a gas vacuolate bacterium, strain S51-W(gv)1 (Gosink & Staley, 1995), isolated from Antarctica. However, this strain has not yet been identified at the species level. Strain ABE-1T also clustered with amplified 16S rRNAs of AGG53 (DeLong et al., 1993) and PVB OTU12 (Moyer et al., 1995) (data not shown) which were isolated from sea macroaggregate and a hydrothermal vent system, respectively. The 16S rRNA sequence of strain ABE-1T exhibited high similarity to that of AGG53 (95.3%) and PVB OTU12 (92.9%).

DNA base composition and DNA–DNA hybridization

The DNA G+C content of strain ABE-1T was 39.4 mol%, a value that falls within the definition range of the genera Colwellia and Vibrio. According to 16S rRNA sequence analysis, strain ABE-1T was closely related to the genus Colwellia. Only two species belonging to genus Colwellia, C. psychrerythraea and Colwellia hadaliensis, have previously been recognized. However, the type strain of C. hadaliensis is difficult to obtain. Therefore, the level of DNA relatedness between strain ABE-1T and only obtainable species, C. psychrerythraea ATCC 27364T, was determined; this value was 22%.

DISCUSSION

Phenotypic characterization and chemotaxonomic analysis of strain ABE-1T in this study were comparable to previous results (Okuyama et al., 1990; Takada et al., 1979, 1989b), except for the casein hydrolysis and DNA G+C content results. Strain ABE-1T was tentatively identified as belonging to the genus Vibrio, due to the characteristic phenotypic similarities to Vibrio marinus, although it has been reported as being negative for fermentation of D-glucose (Takada et al., 1979). In the present study, in addition to the phenotypic characteristics, the almost complete sequence of the 16S rRNA was determined. Phylogenetic analysis based on the 16S rRNA sequence revealed that strain ABE-1T was more closely related to Colwellia species than to Vibrio species (Fig. 2). These results suggest that strain ABE-1T should be classified as Colwellia rather than Vibrio.

Although phylogenetic analysis has revealed that the genera Colwellia and Vibrio are very different (Fig. 2) and strain ABE-1T and C. psychrerythrae do not ferment D-glucose, Colwellia species have been described in the genus Vibrio in terms of phenotypic characterization (Holt et al., 1994). Actually, the Colwellia strains, C. psychrerythrae and C. hadaliensis, have been reported as facultatively anaerobic bacteria (D’Aoust & Kunshner, 1972; Deming et al., 1988), but in this study, generally (four-fifths of trial), C. psychrerythrae did not produce acids from D-glucose under either aerobic or anaerobic conditions during 1 month. However, acids were occasionally produced under aerobic conditions (one-fifth of trial). These results suggested that phenotypic characteristics of the genus Colwellia may also be totally different from

![Fig. 1. Electron micrograph of negatively stained cells of strain ABE-1T showing curved rod and polar flagellum. Bar, 1 μm.](image-url)
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from four other genera of halophilic marine microorganisms on the basis of phenotypic and chemotaxonomic characteristics as follows: genera *Vibrio*, *Photobacterium* and *Moritella* are facultatively anaerobic; and the genus *Halomonas* exhibits a G+C content of DNA of 52–68 mol% and the major isoprenoid quinone detected is Q-9. However, it is very difficult to differentiate between the genus *Colwellia* and the genera *Alteromonas*, *Pseudoalteromonas*, *Shewanella* and *Marinomonas* using these characteristics. In some cases, it is possible to differentiate between strain ABE-1T, which belongs to the genus *Colwellia*, and the cold-adapted strains that belong to these other genera on the basis of phenotypic and chemotaxonomic characteristics. For example, recently, *Pseudoalteromonas antarctica* was isolated from an Antarctic coastal environment (Bozal et al., 1997). Strain ABE-1T was differentiated from *P. antarctica* in terms of cell shape, growth temperature range (4–30 °C), utilization and metabolism of substrates, nitrate reduction and starch hydrolysis.

The available data of phenotypic characteristics of the already known *Colwellia* species, *C. psychrerythraea* and *C. hadaliensis*, are limited (D'aoust et al., 1972; Deming et al., 1988). Strain ABE-1T can be differentiated from the other two reported *Colwellia* species, *C. psychrerythraea* produces red pigment, hydrolyses starch and has a growth temperature range of 0–19 °C; *C. hadaliensis* hydrolyses chitin, is obligately barophilic and has a DNA G+C content of 45.7 mol%. The similarity value between strain ABE-1T and *C. psychrerythraea* was 92.9% on the basis of 16S rRNA sequencing, and the DNA–DNA relatedness value between strain ABE-1T and *C. psychrerythraea* was 22%. These values also indicate that these two strains do not belong to the same species.

*Colwellia*-like gas vacuolate bacterium strain S51-W(gv)1 (Gosink & Staley, 1995), from the Antarctic sea, exhibits a high 16S rRNA sequence similarity value with strain ABE-1T (97.8%). Strain ABE-1T can

...those of the genus *Vibrio*. It is considered that there are several difficulties in judging Leifson's modified oxidation/fermentation test (Leifson, 1963) due to the long period of incubation at low temperature when studying psychrophiles. Interestingly, the *Colwellia* species are more closely related to *Alteromonas macleodii* (Fig. 2), a strictly aerobic marine bacterium, than to facultative anaerobes, such as the genera *Vibrio*, *Photobacterium* and *Aeromonas*, on the basis of phylogenetic analysis. From the results described above, it is concluded that strain ABE-1T belongs to the genus *Colwellia*, and that the definition of *Colwellia* should be amended in terms of d-glucose metabolism.

The phenotypic characterization of halophilic genera that commonly occur in marine habitats is shown in Table 1. The genus *Colwellia* can be differentiated

![Fig. 2. Phylogenetic tree derived from 16S rRNA sequence data of strain ABE-1T and other species within the gamma 3 subclass of the class Proteobacteria. Marinomonas vaga was used as outgroup for the gamma 3 subclass of Proteobacteria. Bar, 0.01 kmol.](image)

Table 1. Characteristics of genus *Colwellia* and genera that commonly occur in marine habitats

<table>
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<tr>
<th>Characteristic</th>
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<th>6</th>
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<th>9</th>
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<td>Na⁺ requirement for growth</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Major isoprenoid quinone(s)</td>
<td>Q-8, MK-8, Q-8, Q-9</td>
<td>ND</td>
<td>ND</td>
<td>Q-8</td>
<td>Q-8</td>
<td>MK-7, Q-7, Q-8</td>
<td>Q-9</td>
<td>Q-8</td>
<td></td>
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<td>Marine inhabitant</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>DNA G+C (mol%)</td>
<td>39-46</td>
<td>38-51</td>
<td>40-44</td>
<td>42-2</td>
<td>44-47</td>
<td>37-50</td>
<td>44-47</td>
<td>52-68</td>
<td>44-48</td>
</tr>
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</table>

Data were obtained from the following references: Akagawa-Matsushita et al. (1992); Baumann et al. (1984); Collins & Jones (1981); D'aoust & Kunshner (1972); Deming et al. (1988); Dobson & Franzmann (1990); Gauthier et al. (1995); MacDonell & Colwell (1985); and Van Landschoot & De Ley (1983).
Acids are produced from D-glucose under aerobic conditions; however, no acids are produced in an-flagellum. Catalase and oxidase reactions are positive. The strain grows in the absence of NaCl in the culture medium, but growth occurs in media supplemented with 3 and 4% NaCl and not in media with salinity higher than 6.5% NaCl. Susceptibility to vibriostatic compound O/129 (10 and 150 µg) is observed. Growth occurs at 0-22°C, but not at temperatures higher than 25°C. Voges–Proskauer test, methyl red test and indole production are negative, but reduction of nitrate is positive. Hydrolyses gelatin, methyl red test and indole production are negative, but growth is observed in the absence of NaCl in the culture medium, but growth occurs in media supplemented with 3 and 4% NaCl and not in media with salinity higher than 6.5% NaCl. Susceptibility to vibriostatic compound O/129 (10 and 150 µg) is observed. Growth occurs at 0-22°C, but not at temperatures higher than 25°C. Voges–Proskauer test, methyl red test and indole production are negative, but reduction of nitrate is positive. Hydrolyses gelatin, DNA and Tweens 20, 40, 60 and 80, but does not hydrolyse casein, chitin, starch and algic acid. Utilizes D-glucose as a sole carbon and energy source for growth, but not D-mannose, raffinose, D-xylose, L-arabinose, D-fructose, glycerol, lactose, maltose, melibiose and sucrose. The major isoprenoid quinone is Q-8. The whole-cell fatty acids contain saturated and monounsaturated fatty acids with 10–18 C atoms; saturated and mono-unsaturated C16 fatty acids are predominant in cells grown at 15°C. The strain contains a unique trans-unsaturated fatty acid (9-trans-hexadecenoic acid, C16:19t). The DNA G+C content is 39.4 mol% (determined by HPLC). The type strain of Colwellia maris is ABE-1\textsuperscript{T} (= JCM 10085\textsuperscript{T}).

**ACKNOWLEDGEMENTS**

The authors would like to thank Dr Y. Noda (Hokkaido University) for his help with transmission electron microscopic observation and Dr K.-I. Suzuki (Japan Collection of Microorganisms, Institute of Physical and Chemical Research) for critically reading the manuscript.

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