

PHYLOGENETIC CHARACTERIZATION OF PSYCHROPHILIC BACTERIA ISOLATED FROM DEEP-SEA WATER OF THE NANKAI TROUGH

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ABSTRACT

A screening among 4°C-culturable isolates obtained from deep-sea water of Nankai Trough from a depth of approximately 4,000 m resulted in a total of 5 psychrophilic bacteria showing no growth at 20°C. Phylogenetic characterization based on 16S ribosomal DNA sequence analysis showed that the isolates belonged to the genus *Colwellia*, *Moritella*, and *CNPT-3*, which could be assigned to *Psychromonas*.

Keywords: Marine psychrophiles, 16S rDNA, *Colwellia*, *Moritella*, *Psychromonas*

I. INTRODUCTION

Deep-sea is a region commonly characterized by extremely low temperatures and with the exception in the vicinity of hydrothermal vents, deep-sea temperatures are close to 0 °C and very stable. Thus, microorganisms living in deep-sea areas would manifest adaptation to permanently low temperatures.

The interest in low temperature adapted bacteria particularly psychrophilic bacteria, originated from different considerations, i.e. potential biotechnological implications (Gounot, 1991; Ohgiya *et al.*, 1999); low temperature adaptation (Hamamoto *et al.*, 1994); ecology (Kato *et al.*, 1995; Bowman *et al.*, 1997), and

phylogeny (DeLong *et al.*, 1997; Knoblach *et al.*, 1999).

DeLong *et al.*, (1997) reviewed the phylogenetic relationships of cultivated deep-sea psychrophilic and barophilic bacteria, and demonstrated that these bacteria were affiliated with one of five genera within the gamma subclass *Proteobacteria*: *Colwellia*, *Shewanella*, *Photobacterium*, *Moritella* and *CNPT-3*, which is regarded as unidentified group. Since then, studies on new finding and description of deep-sea bacteria have been reported (Nogi *et al.*, 1998a, 1998b, 1998c; Kato and Qureshi, 1999).

However, no isolation and characterization of psychrophilic bacteria from Nankai Trough has been so far

reported. It is believed that this is the first report on phylogenetic characterization of psychrophilic bacteria isolated from the permanently cold seawater of Nankai Trough (33° 30.0'N, 137° 53.6 E).

II. MATERIALS AND METHODS

2.1. Sampling protocols

Seawater samples just above sediment were collected using Niskin butterfly water samplers (General Oceanics, Florida) during KT-99-9 cruise from 25 June to 2 July, 1999 by R/V Tansei Maru, Ocean Research Institute, University of Tokyo.

Once arrived onboard, seawater samples were then immediately transferred to sterile glass bottles on ice. Nine ml seawater samples were aseptically poured into pre-cooled sterile plastic tubes, and were added with 1 ml pre-cooled ZoBell 2216E. Sample preparations were carried out under 10 °C condition to avoid thermal shocks. All tubes were then incubated at 4 °C for 30 d. Pure cultures were obtained by streak method on 1/5 strength ZoBell 2216E under 4 °C incubation.

2.2. Growth studies

Screening of psychrophiles among deep-sea strains was based on the ability of strains to grow at 4 °C but not at 20 °C incubation (Morita, 1975) on ZoBell 2216E agar medium. Barophily test was performed by inoculating 20 l cultures into 3 ml 1/5 strength ZoBell 2216E broth medium in small sterile polyethylene bags (Whirl-Pak, Nasco, USA) which were sealed with heat sealer and were incubated for 10 d at 4 °C at atmospheric and *in situ* pressure (40 MPa). Growth was measured by using a spectrophotometer (Spectronic 21, Milton Roy, USA) at 600 nm at the beginning and the end of incubation.

2.3. DNA extraction and PCR amplification

Cells were harvested by centrifugation from cultures grown at 4 °C on ZoBell 2216E broth medium according to Radjasa et al. (2001a). DNAs were extracted with proteinase K (1 mg ml⁻¹) (Sigma Chemical Co, St. Louis, MO, USA), 50 l PCR reaction mixtures were prepared and 30 cycles amplifications were performed as described by Urakawa et al., (1997). Amplified DNA was examined by 1% agarose gel in TAE electrophoresis buffer with 1 l aliquots of PCR product.

2.4. DNA sequencing and phylogenetic analysis

PCR products were purified using Microcon-100 micro-concentrators (Amicon, Beverly, MA, USA) and were prepared using a SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) for subsequent sequencing on an automated sequencer (Pharmacia LKB Biotech, Uppsala, Sweden) as previously described (Radjasa et al, 2001b). The resulting rDNA sequences were compared with the database using BLAST and FASTA searches.

Sequences were aligned to the nearest neighbour using The CLUSTAL W program (Thompson et al. 1994), and were then manually aligned using MacClade program (Ver.3.06). The phylogenetic tree was constructed based on Neighbour-joining method (Saitou and Nei 1987). Bootstrap confidence analysis was carried out on 1000 resamplings.

III. RESULTS AND DISCUSSION

Five isolates among bacterial isolates (grown at 4 °C) from seawater of the Nankai Trough at a depth of 4,000 m were able to grow well at 4 °C, but were

unable to grow at 20 °C (Table 1). It is indicating that they were psychrophilic types (Morita, 1975). Deep-sea water of the Nankai Trough could be regarded as permanently cold marine environment since the observed environmental temperatures were below 5 °C. In a recent study, Li *et al.*, (1999) detected a predominance of mono unsaturated fatty acids mainly 18:1 isomers in the sediments of cold seeps of the Nankai Trough which can be interpreted as a response to low temperature and a predominance of psychrophilic bacteria. The present study clearly supported their results.

In contrast with shallow-living psychrophiles, deep-sea psychrophiles also encounter another environmental difference, i.e. hydrostatic pressure. Usually the growth of various organisms are inhibited by pressure in the range of 30 – 50 MPa (Abe *et al.*, 1999), so that adaptation to high pressure as well as to low temperature would also be expected to be specific characteristics among deep-sea psychrophiles from the Nankai Trough. Our isolates were found to tolerate the pressure of their capture depth of 40 MPa (Table 1), and therefore were regarded as barotolerant psychrophilic bacteria (Kato *et al.*, 1995).

The most common strategy employed by psychrophilic or barophilic bacteria to survive in low temperature and high pressure environments is the high percentages of long-chain polyunsaturated fatty acids (PUFAs) in the membrane lipids (DeLong and Yayanos, 1986; Wirsen *et al.*, 1987), including eicosapentaenoic acid (EPA) in *Photobacterium* (Nogi *et al.*, 1998a), *Shewanella* (DeLong *et al.*, 1997; Nogi *et al.*, 1998b; Kato *et al.*, 1998);

docosahexaenoic acid (DHA) among the genus *Moritella* (DeLong *et al.*, 1997; Kato *et al.*, 1998; Nogi *et al.*, 1998c) and *Colwellia* (DeLong and Yayanos, 1986; Bowman *et al.*, 1998).

The identification of 16S rDNA sequences of each isolate is summarized in Table 1. The phylogenetic position of the 16S rDNA sequences of psychrophilic bacteria isolated from seawater is shown in Fig. 1. Among seawater isolates, 16S rDNA sequencing of NTW-1, NTW-3, and NTW-4 showed high homology to members of the genus *Colwellia* (97.0%-98.0%). *Colwellia* species appear to be widely distributed in coastal and abyssal oceanic regions characterized by permanently low temperature and/or high-pressure marine environments (Bowman *et al.*, 1998). Among seven reported species of the genus *Colwellia* (Deming *et al.*, 1988; Bowman *et al.*, 1998; Yumoto *et al.*, 1998), only *C. hadaliensis* and *C. psychroerythrus* have been isolated from deep-sea waters. Thus it is not surprising that the seawater isolates in this study showed close relationship to *C. psychroerythrus* as well.

Interestingly, NTW-2 isolate showed an affiliation with the unidentified group consisting of CNPT-3 and WHB46-2 (DeLong *et al.*, 1997). Isolate CNPT-3 is a facultative barophilic bacterium originated from a depth of 5.700 m in the central North Pacific Ocean. On the other hand, WHB46-2 is an Antarctic barophile from the Weddell Sea (Liesack *et al.*, 1991). Since both isolates have been assigned to unidentified group by DeLong *et al.*, (1997), there has been no further report on the isolation of deep-sea barophilic or psychrophilic bacteria related to this group.

Table 1. Characteristics of seawater psychrophiles from the Nankai Trough

Isolate	Psychrophily		Barophily	Closest species	Similarity %
	4°C	20°C	40Mpa		
NTW-1	+	-	+	<i>Colwellia psychroerythrus</i>	97.0
NTW-2	+	-	+	<i>Psychromonas</i> sp.	96.0
NTW-3	+	-	+	<i>Colwellia psychroerythrus</i>	97.6
NTW-4	+	-	+	<i>Colwellia psychroerythrus</i>	98.0
NTW-5	+	-	+	<i>Moritella japonica</i>	96.8

The unexpected result was when closely related sequences of NTW-2 deduced from database were included, CPNT-3, WHB64-2, and NTW-2 were clearly clustering within the genus *Psychromonas* (Fig.1). The type species of this genus is *Psychromonas antarcticus*, a aerotolerant anaerobic, halophilic psychrophiles isolated from pond sediment of the McMurdo Ice Shelf, Antarctica (Mountfort *et al.*, 1998). The percentage of similarities of NTW-2 with its closest neighbors is listed in Table 2. NTW-2 showed a highest similarity to the sequence of *Psychromonas* sp., a psychrophile isolated from surface seawater in winter

time at the Okhotskoe Sea when floating sea ices coming to Northern Hokkaido, Japan (Yumoto, *personal communication*).

Table 2. Percent similarities of NTW-2 to its closest neighbors

Neighbor	Similarity (%)	Source
<i>Psychromonas</i> sp.	96.0	Hokkaido, Japan
CNPT-3	96.0	North-western Pacific
<i>Psychromonas antarcticus</i>	95.0	McMurdo, Antarctica
<i>Psychromonas</i> sp. IC004	95.0	Coastal Antarctica

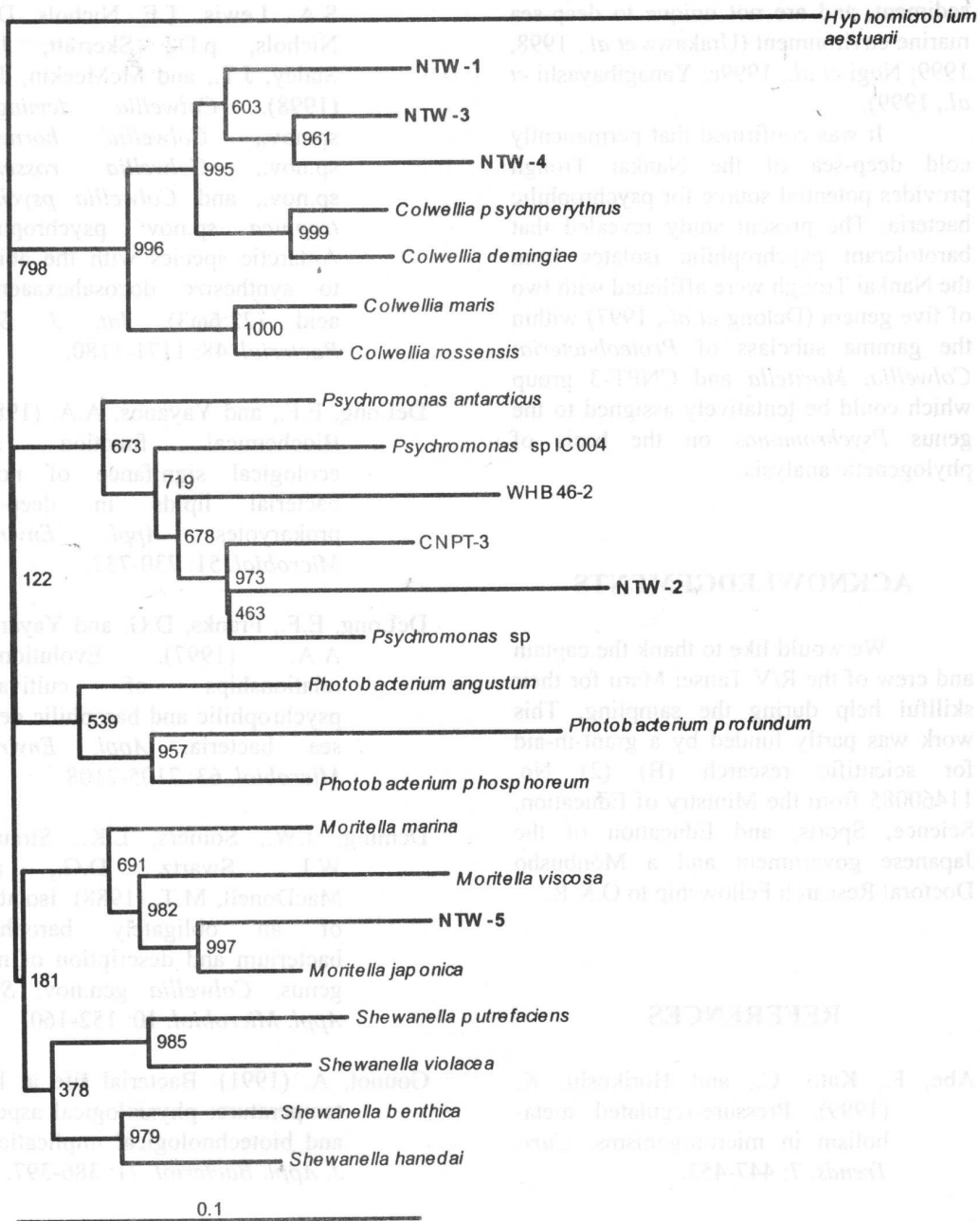


Fig 1. Phylogenetic affiliation of seawater isolates of the Nankai Trough. *Hypomicrobium aestuarii* was used as out-group. Bar indicates 10% sequence dissimilarities.

Seawater isolate NTW-5 was mostly related to *Moritella japonica*, a moderately barophilic bacterium isolated

from the Japan Trench sediment at a depth of 6356 m (Nogi *et al.*, 1998c). The members of the genus *Moritella* have been

commonly found both at seawater and sediment, and are not unique to deep-sea marine environment (Urakawa *et al.*, 1998, 1999; Nogi *et al.*, 1999c; Yanagibayashi *et al.*, 1999).

It was confirmed that permanently cold deep-sea of the Nankai Trough provides potential source for psychrophilic bacteria. The present study revealed that barotolerant psychrophilic isolates from the Nankai Trough were affiliated with two of five genera (DeLong *et al.*, 1997) within the gamma subclass of *Proteobacteria*: *Colwellia*, *Moritella* and CNPT-3 group which could be tentatively assigned to the genus *Psychromonas* on the basis of phylogenetic analysis.

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