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— Original Paper —

## Isolation and characterization of biodegradable plastic degrading bacteria from deep-sea environments

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We have isolated thirteen different bacterial strains as poly  $\epsilon$ -caprolactone (PCL)-degrading bacteria from the Kurile and Japan Trenches at a depth of 5,000-7,000 m (deeper ocean bottoms). The isolates belong to the *Shewanella*, *Moritella*, *Psychrobacter* and *Pseudomonas* genera. This is the first record of PCL degrading bacteria isolated from deep-sea environments at depth of over 5,000 m. Six strains of the isolates, numbered CT01 in genus *Shewanella*, CT12, JT01 and JT04 in genus *Moritella*, JT05 in genus *Psychrobacter*, and JT08 in genus *Pseudomonas* were selected for investigation of their cell shapes, degrading abilities for several aliphatic polyesters, and growth profiles. The cell shapes of the strains, except JT05, were rod-shaped, non-spore-forming and motile by means of a single or multi polar flagella. The cell shapes of JT05 were coccal with no visible flagella. From the results of degradation tests on six different aliphatic polyesters, all strains could degrade only PCL. Strains CT01, CT12, JT01 and JT04 are psychrophilic and pressure tolerant bacteria and three strains except JT04 showed typical piezophilic growth profiles. Therefore, it is possible that these strains might play a role in degrading aliphatic polyesters under deep-sea conditions, ie., low-temperatures and high hydrostatic pressures.

**Keywords:** aliphatic polyester, biodegradation, deep-sea, piezophilic bacteria, plastic degrading bacteria

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## 1. Introduction

Plastic wastes are one of the factors in causing environment pollution, because of their semi-permanent stability in the environment. According to a National Academy of Sciences report, approximately  $4.5 \times 10^4$  metric tons of plastic are discarded to the ocean annually (Edward, 1975). Therefore, the ocean is a common environment influenced by plastic pollution. Specifically, the deep-sea floor has the potential of being the final site for plastic wastes. Actually, abundant plastic wastes have often been found during deep-sea investigations using research submersibles (Fujikura et al., 2008). Partial replacement of non-degradable plastics with biodegradable polymers is one strategy for reducing the negative environmental impact caused by plastic materials since the biodegradable polymers can be degraded by microorganisms (Tabata and Kanehiro, 2004). Several biodegradable plastics, such as poly  $\beta$ -hydroxybutyrate (PHB), poly  $\epsilon$ -caprolactone (PCL), poly ethylene succinate (PES), poly butyrene succinate (PBS) and poly lactic acid (PLA), have been discovered (Tokiwa and Calabia, 2004).

From deep-sea environments, numerous cold and high-hydrostatic pressure adapted microorganisms, called piezophiles, have been isolated and characterized (Alain et al., 2002; Kato et al., 1995; Kato et al., 1998; Kato et al., 2008; Nogi et al., 1998a; Nogi et al., 1998b; Nogi et al., 1999; Nogi et al., 2002; Nogi et al., 2004; Nogi et al., 2007; Xu et al., 2003; Yanagibayashi et al., 1999; Yayanos, 1995). These bacteria are able to survive at low temperature and high-pressure conditions. The current research focus is on the interactions between the deep-sea environment and its microbial inhabitants. However, there is no current information related to screening for useful piezophilic bacteria. In addition, it can be assumed that plastic degrading bacteria in the deep-sea environments might also be piezophiles.

In our previous study, we isolated PCL-degrading bacteria from deep seawaters at depth of 300-600 m and investigated their cultural characteristics and relative abilities to biodegrade several aliphatic polyesters (Sekiguchi et al., 2009). However, biodegradable plastic degrading activities of the microorganisms living at the deeper sea bottoms are still unknown. In this study, to obtain proof for plastic biodegradation in these environments, we have attempted to isolate and characterize aliphatic polyester degrading piezophilic and psychrophilic bacteria from Kurile and Japan Trenches.

## 2. Materials and Methods

### 2.1. Collection of deep-sea sediment samples

Deep-sea sediment samples were collected by the submersibles *SHINKAI 6500* (dive No. 6K#1029, at a depth of 4,819 m, September 2007, during the cruise YK07-14, PI: T. Miwa) and *KAIKO 7000II* (dive No. 7K#399, depth of 5,356 m, and dive No. 7K#400, depth of 6,957 m, November 2007, during the cruise KR07-14, PI: C. Kato), in the Kurile and Japan Trenches using sediment samplers. Sampling sites and photographs are shown in Fig. 1. These sampling sites were areas enriched with *Calyptogen* communities. The sediments suspended with artificial seawater were added into each 2 ml sterilized tube with 1 cm square PCL films (Sigma Aldrich co., Japan) sterilized with 70% ethanol and then sealed with parafilm immediately after being taken from the deep-sea. Each plastic tube was statically placed in a pressure vessel controlled at constant temperature ( $4^\circ\text{C}$ ) and pressure (50 MPa) conditions. These operations were carried out on ice. The sediments were kept under these conditions until microbial growth was confirmed.

### 2.2. Confirmation of growth of microorganisms

The sediment maintained at 50 MPa in pressure vessels was observed with a fluorescence and phase contrast microscopy system (Olympus Co., Japan) for confirmation of microbial growth. The cells were stained with diamidino -2-phenylindole (DAPI).

### 2.3. Isolation of piezophilic PCL-degrading bacteria

The screening procedure for PCL-degrading bacteria from the deep-sea environments using a PCL-granule agar media and high-pressure (HP) cultivation (DEEPBATH) was described by Sekiguchi et al. (2010). The medium of 1.0 L contained: 1.87% MB, 1.5% NaCl, 0.35% KCl, 5.4%  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 2.7%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . The pressurized the deep-sea sediments cultivated three times in the DEEPBATH system with PCL films at  $4^\circ\text{C}$  and 50 MPa until confirmed microorganisms growth following inoculation and then added onto PCL-granule agar media. The plates were incubated at  $4^\circ\text{C}$  and 50 MPa for one week. Bacterial degrading ability was determined by the formation of clear zones around the colonies on the PCL-containing agar plates. The colonies forming clear zone were inoculated onto fresh PCL agar-plates. The PCL-degrading bacteria were isolated by repeating this operation twice or three times until a single

colony was obtained.

#### 2.4. Extraction of DNA from isolates and the sequence analysis

Extraction of chromosomal DNA from isolates was conducted according to the method of Saito and Miura (1963). 16S rRNA genes were amplified using the PCR method with primers Eubac27F and Eubac1492R (DeLong, 1992). The amplified DNA was sequenced by a direct sequencing procedure to determine species relatedness. Nucleotide-substitution rates (K<sub>nu</sub>c) (Kumira, 1980) were also determined and phylogenetic tree was constructed by using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with the CLUSTAL W program (Thompson et al, 1994). Alignment gaps and unidentified base positions were not utilized in the calculations. The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1000 replicates. The DNA sequences accession numbers reported in this paper were AB554717 to AB554729.

#### 2.5. Morphology of the isolates by electron microscopy

The morphology of isolated cells was observed by transmission electron microscopy (TEM). For negative staining, 1 drop of culture was placed on a copper grid coated with Pioloform and carbon followed by staining 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.

#### 2.6. Evaluation of the degradability of other aliphatic polyesters by the clear-zone method

The isolates were grown on agar medium containing 1% (w/v) of PCL, PBS, PBSA, PHB/V (polyhydroxyl butyrate/valerate) or PLA granules. Bacterial degrading ability was determined by the formation of clear zones around the colonies on the aliphatic-polyesters-containing agar plates containing 1.0% aliphatic polyesters, 1.87% MB, 1.5% NaCl,

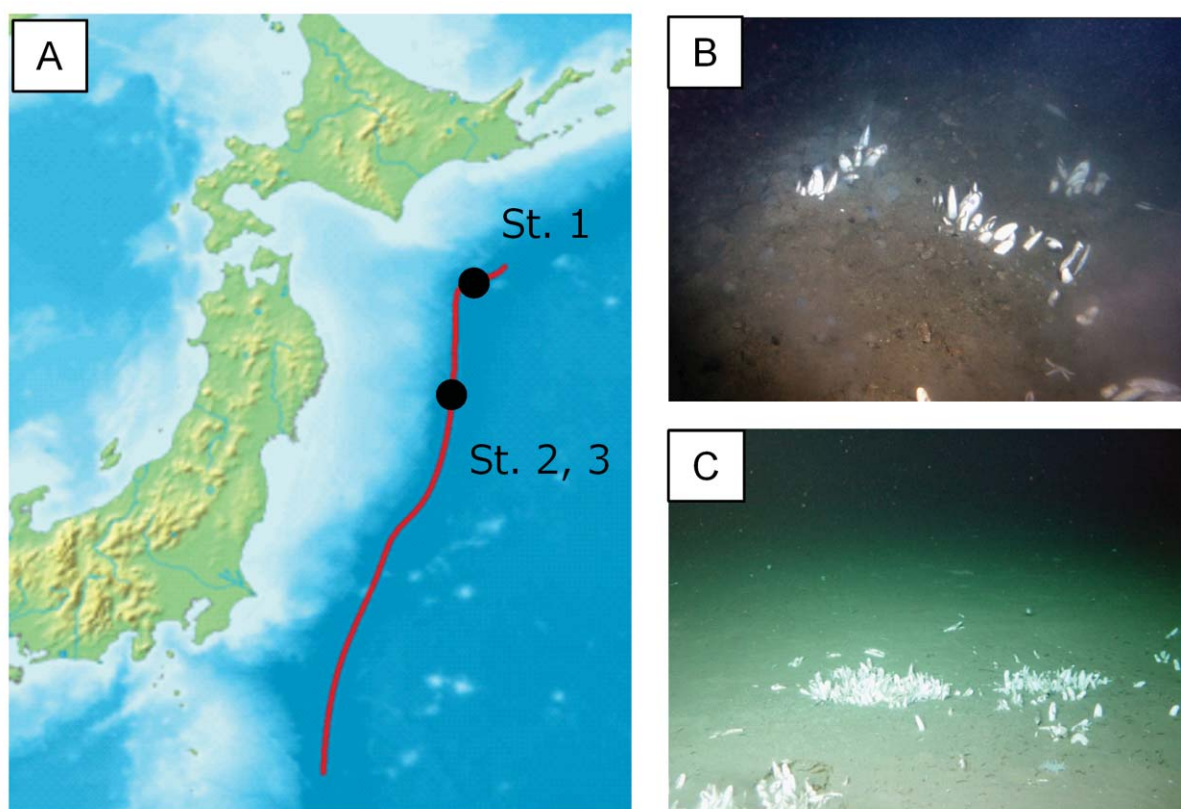


Fig. 1. (A) Location of the sampling points for deep-sea sediments used in this study. Station 1 (St.1): Kurile Trench at a depth of about 5,000 m (dive No. 6K#1029, 41). Stations 2 and 3 (St.2, St.3): Japan Trench at a depth of 5,000 and 7,000 m (dives No. 7K#399, 39 and 7K#400, 40), respectively. (B) Site photo of station 1. (C) Site photo of station 2.

0.35% KCl, 5.4% MgCl<sub>2</sub>·6H<sub>2</sub>O, 2.7%, MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.5% CaCl<sub>2</sub>·2H<sub>2</sub>O. Cultivations were performed for 1 month.

### 2.7. Optimal growth conditions

Optimal temperatures for the growth of isolates were investigated in the range of 4–20°C on the basis of optical density in a 1/2MB liquid media containing 1.87% MB, 1.5% NaCl, 0.35% KCl, 5.4% MgCl<sub>2</sub>·6H<sub>2</sub>O, 2.7%, MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.5% CaCl<sub>2</sub>·2H<sub>2</sub>O. High-pressure cultivation for determination of optimal pressure for growth was carried out using the modified method of Kato *et al.* (2006). About 2.0 ml of the liquid media inoculated with the isolates and 500 µl per-fluorinated liquids (Sumitomo-3M Co., Japan) saturated with O<sub>2</sub> were added into each sterilized plastic tube and sealed with parafilm. All procedures were carried out on ice. For strains CT01, CT12, JT01 and JT04, each plastic tube was placed in a titanium pressure vessel filled with water at 8°C. In the case of strains JT05 and JT08, the water temperature was maintained at 25°C. The pressures in the vessels were controlled at constant pressures of 0.1, 10, 30, 50 and 70 MPa. To evaluate the growth of the isolates under these conditions, the medium was taken out from each vessel every 12 hours and the OD value of the medium at 660 nm was measured using a spectrophotometer (Ultraspec 500 pro, Amersham GE Healthcare, Japan).

## 3. Results and Discussion

### 3.1. Isolation of piezophilic PCL-degrading bacteria

The sediments obtained from the Kurile and Japan Trenches at depth of 5,000–7,000 m were maintained at 4°C, 50 MPa with PCL films in the pressure vessels for isolation of HP adapted PCL-degrading microorganisms. Two weeks later, bacterial growth was confirmed around PCL films (Fig. 2). Sediment fluids positive for bacterial growth were inoculated for secondary HP cultivation using the DEEPBATH system. Next, the HP mixed cultivation fluids were placed onto agar plates containing 1% PCL granules at 4°C. After several days, bacterial colonies with clear zones (halo-formations) were observed on the PCL-granules agar plates (Fig. 3). We also confirmed halo-formation under high-pressure conditions using the same medium (Sekiguchi *et al.*, 2010). A total of thirteen different PCL-degrading bacterial strains were identified based on the 16S rRNA gene sequences comparisons. A phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 4. Eight strains of these isolates, CT05, CT06, CT08, CT12, CT13, JT01, JT02 and JT04, were closely related to *Moritella* sp. and three strains, CT01, CT03 and CT07, were closely related to *Shewanella* sp., respectively. Strains JT05 and JT08 were closely related to *Psychrobacter* sp., *Pseudomonas* sp., respectively. These related species have not been previously reported as aliphatic-polyesters-degrading bacteria. Thus, this is the first report, which confirms that PCL-degrading bacteria are distributed in deep-sea environments at depth of over 5,000 m and they could play a role in degrading aliphatic polyesters under deep-sea, low temperature and high-pressure conditions.

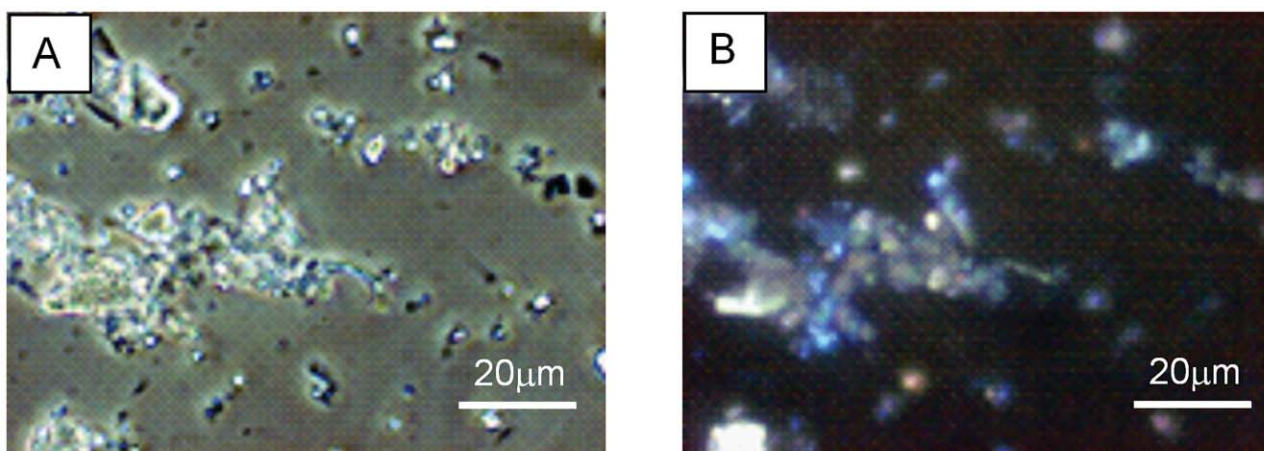


Fig. 2. Observations of microorganisms growth with PCL films under conditions of 4°C and 50 MPa for 2 weeks. A: Bright-field image. B: Fluorescence image stained with DAPI.

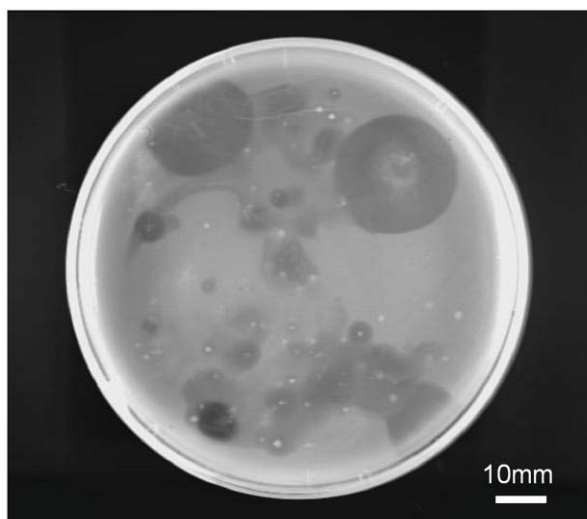


Fig. 3. Clear-zone formation by PCL-degrading bacteria from the Kurile Trench on agar plates containing 1% (w/v) PCL granules.

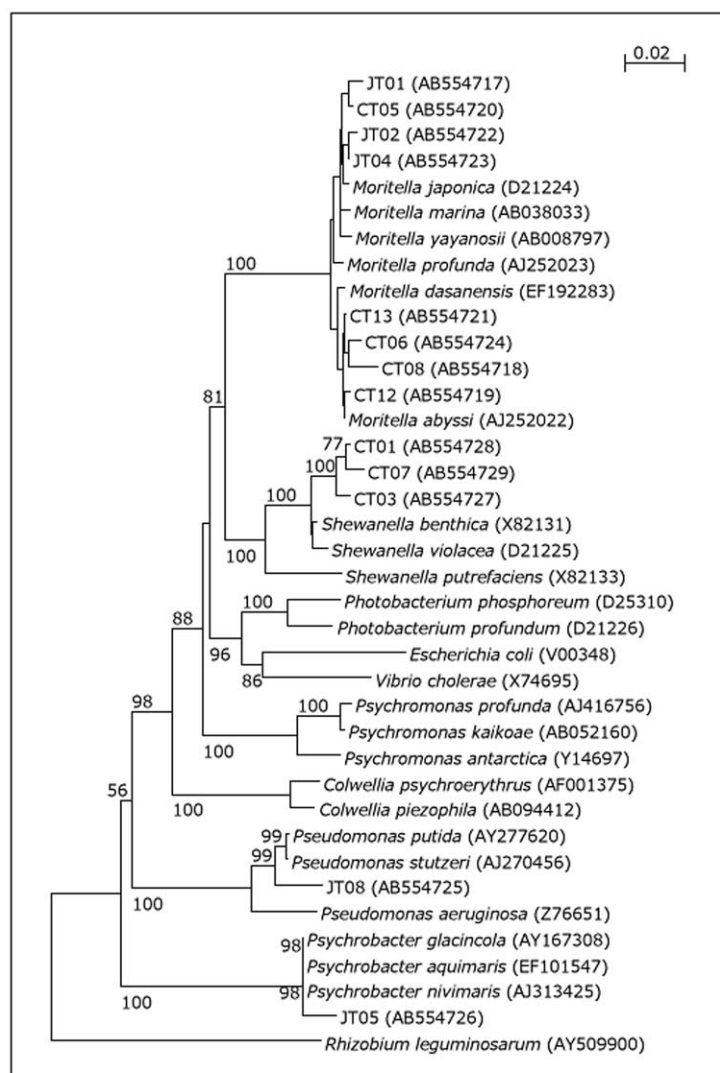


Fig. 4. Phylogenetic tree showing the relationship between isolated deep-sea bacterial strains within the gamma-Proteobacteria subgroup based on 16S rRNA gene sequences with the neighbor-joining method. The scale represents the average number of nucleotide substitution per site. Bootstrap values (%) are shown for frequencies above the threshold of 50%.

### 3.2. Characterization of the isolates

Six strains in the isolates, CT01, CT12, JT01, JT04, JT05 and JT08, were selected based on the phylogenetic relations shown in Fig. 4 and their characteristics were determined.

The cell shapes of the isolates, except JT05, were rod-shaped, non-spore-forming and motile by means of a single or multiple unsheathed, polar flagella (Fig. 5). Specifically, strain JT01 had multiple polar flagella, whereas other *Moritella* species had a single polar flagellum. For strain JT05, cell shapes were coccid and no visible flagella.

The degrading activities with several aliphatic-polyesters, PCL, PHB, PBS, PBSA and PLA, were investigated for these isolates with the clear-zone methods (Table 1). According to the isolation procedure, all of the isolates were able to degrade the PCL material as a substrate. However, all other aliphatic-polyesters, PHB, PBS, PBSA and PLA, could not be degraded following one month cultivation. In this study, thirteen PCL degrading bacteria were isolated from the deep-sea sediments. However, isolations of other aliphatic-polyesters-degrading bacteria are necessary for evaluation of biodegradability of biodegrading plastics under deep-sea environments, because these conditions (low temperature and high-pressure conditions) are very different compared to land

and compost ones.

The growth characteristics of the six selected strains are shown in Table 2. For strains CT01, CT12 and JT01, the highest growth rate in 1/2MB was recorded at 5-8°C, but no growth was observed above 15°C. In the case of strains JT05 and JT08, optimal growth temperatures were recorded at 32 and 35 °C, respectively. Furthermore, optimal pressures for growth of strains CT01, CT12 and JT01 ranged from 30-50 MPa. For strain JT04, the highest growth rate was recorded under atmospheric pressure but the strain can grow under very high hydrostatic pressure conditions, i.e., 70 MPa. On the other hand, for strains JT05 and JT08, optimal pressure conditions for growth were at atmospheric pressure and they were not able to grow at pressures greater than 50 MPa. These results indicated that strains CT01, CT12 and JT01 are psychrophilic and piezophilic bacteria, strain JT04 is a psychrophilic and piezotolerant bacterium, and strains JT05 and JT08 are mesophilic and piezosensitive bacteria. Therefore, strains JT05 and JT08 might not be functioning as aliphatic-polyesters-degrading bacteria at deep-sea environment. We suggested that the isolated piezophilic and piezotolerant bacteria (strains CT01, CT12, JT01 and JT04) could degrade aliphatic polyesters, at least PCL, at the deep-sea bottoms.

Table 1. Aliphatic polyester degrading activities of deep-sea isolated strains.

Strains	Clear-zone formation on the plate				
	PCL	PHB	PBS	PBSA	PLA
CT01	+	-	-	-	-
CT12	++	-	-	-	-
JT01	++	-	-	-	-
JT04	+	-	-	-	-
JT05	+	-	-	-	-
JT08	+++	-	-	-	-

-: negative, +, ++ and +++ : relative size of clear-zones around the colonies.

Table 2. Effects of temperature and pressure conditions on the growth of the isolated PCL-degrading strains.

Strains	Temperature range of growth (optimal temp.) (°C)	Pressure range of growth (optimal pressure) (MPa)
<i>Shewanella</i> sp. CT01	0-12 (8)	0.1-70 (30)
<i>Moritella</i> sp. CT12	0-12 (5)	0.1-70 (50)
<i>Moritella</i> sp. JT01	0-12 (5-8)	0.1-70 (30)
<i>Moritella</i> sp. JT04	4-15 (12)	0.1-70 (0.1)
<i>Psychrobacter</i> sp. JT05	5-35 (32)	0.1-30 (0.1)
<i>Pseudomonas</i> sp. JT08	13-40 (35)	0.1-30 (0.1)

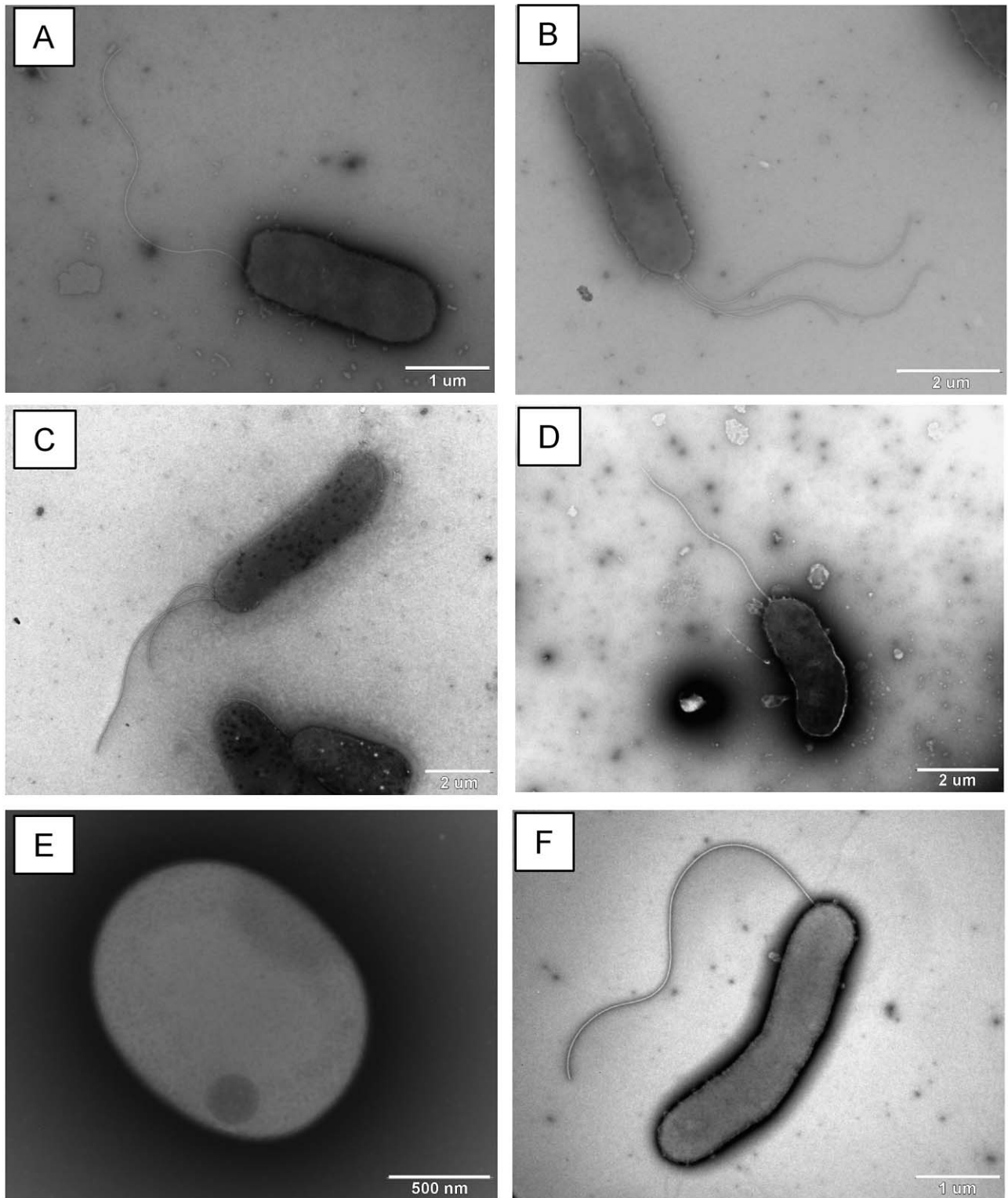


Fig. 5. Electron micrographs of stained shadow-casted cells of the isolated PCL degrading bacteria from deep-sea sediments.  
A:CT01. B:CT12. C:JT01. D:JT04. E:JT05. F:JT08.

#### 4. Conclusions

Thirteen bacterial strains were isolated in total as PCL-degrading bacteria from the sediments collected from the Kurile and Japan Trenches. The isolates belong to the genera *Shewanella*, *Moritella*, *Psychrobacter* and *Pseudomonas*. This is the first report that PCL-degrading bacteria were isolated from deep-sea environments at depth over 5,000 m. From the results of the effects of temperature and hydrostatic pressure on the growth of the isolates, the isolates belonging into the genera *Shewanella* and *Moritella* were shown to be psychrophilic high-pressure adapted bacteria. Therefore it is reasonable to assume that these strains might play a role in degrading aliphatic polyesters such as PCL, at the deep-sea bottoms.

We demonstrated in this study that some biodegradable plastic materials might not be able to be degraded by deep-sea microorganisms. Thus, to identify new biodegradable materials for ocean environments, our isolates could be useful for evaluation of such new materials. Based upon the results of our study, the utilization of aliphatic polyesters in commercial products such as disposable shopping bags and fishing gear might be able to reduce their negative environmental impact particularly at the bottom of the seas.

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