J. Microbiol. Biotechnol. (2006), **16**(11), 1826–1831

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Thermococcus onnurineus sp. nov., a Hyperthermophilic Archaeon Isolated from a Deep-Sea Hydrothermal Vent Area at the PACMANUS Field

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Received: June 2, 2006
Accepted: July 27, 2006

Abstract A novel hyperthermophilic, anaerobic, heterotrophic archaeon, designated strain NA1^T, was isolated from a deep-sea hydrothermal vent area (depth, 1,650 m) within the Papua New Guinea-Australia-Canada-Manus (PACMANUS) field. Cells of this strain were motile by means of polar flagella, coccoid-shaped with a diameter of approximately 0.5–1.0 μm, and occurred as single cells. Optimal temperature, pH, and NaCl concentration for growth were 80°C, 8.5, and 3.5%, respectively. The new isolate was an obligate heterotroph that utilized yeast extract, beef extract, tryptone, peptone, casein, and starch as carbon and energy sources. Elemental sulfur was required for growth and was reduced to hydrogen sulfide. The G+C content of the genomic DNA was 52.0 mol%. Phylogenetic analysis of the 16S rRNA gene indicated that strain NA1^T belongs to the genus *Thermococcus*, and the organism is most closely related to *T. gorgonarius*, *T. peptonophilus*, and *T. celer*; however, no significant homology was observed among species by DNA-DNA hybridization. Strain NA1^T therefore represents a novel species for which the name *Thermococcus onnurineus* sp. nov. is proposed. The type strain is NA1^T (=KCTC 10859, =JCM 13517).

Key words: Archaea, deep-sea hydrothermal vent, hyperthermophile, PACMANUS field, *Thermococcus* sp.

The order *Thermococcales* is represented by three genera: *Thermococcus* [33], *Pyrococcus* [9], and *Palaeococcus* [29], which are distinguished by their optimal growth temperature (80–90°C for *Thermococcus* and *Palaeococcus*; 95–100°C for *Pyrococcus*), 16S rRNA gene sequences, and genomic DNA G+C contents [32]. *Thermococcales* species are strictly anaerobic, obligate heterotrophs that grow preferentially on proteinaceous substrates such as yeast extract, meat extract, peptone, bacterial and archaeal cell homogenates, and polymers such as casein, gelatin, starch, chitin, and xylan as carbon and energy sources. These organisms reduce elemental sulfur as an electron acceptor to remove the formation of molecular hydrogen, which inhibits their growth in closed vessels [24].

Species belonging to the genus *Thermococcus* are found in deep-sea and shallow marine hydrothermal vents [28], freshwater thermal springs [22], and deep oil reservoirs [20]. Most species of *Thermococcus* have very similar physiological and morphological characteristics and can be classified into two distinct groups on the basis of their G+C contents [12].

Recently, the access of unknown prokaryotic diversity in diverse marine environments has been attracted in microbial ecology [16]. Takai *et al.* [30] showed by culture-dependent and independent methods that members of *Thermococcus* were abundant on the chimney surfaces of the Papua New Guinea-Australia-Canada-Manus (PACMANUS) site. Hyperthermophiles have provided the subjects of considerable interests for industrial applications [5, 25]. Here, we describe a novel species of *Thermococcus* isolated from a deep-sea hydrothermal vent area at the PACMANUS field in the Manus Basin near Papua New Guinea.

Sediment samples were collected via a multiple corer, during the "DAE-YANG" cruise (2002) using the vessel *R/V Onnuri* within the PACMANUS hyperthermal field (3°44'S, 151°40'E), at a depth of 1,650 m in the Manus Basin. On board, samples were immediately transferred into an anaerobic chamber and crushed in serum vials filled with 20 ml of sterile reduced artificial seawater, under anaerobic conditions. The serum vials were then closed tightly with butyl rubber stoppers and stored at 4°C until used for further experiments.

Anaerobic procedures were performed as described by Balch and Wolfe [3]. Each sample was used as the inoculum at three different dilutions (1/1, 1/10, and 1/100).

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Enrichment cultures were performed anaerobically in 25-ml serum vials containing 20 ml yeast extract-peptone-sulfur (YPS) medium [14], and incubated in an oven at 80°C for 3 days. Positive cultures were identified by turbidity and microscopic observations. Isolations were performed by streaking positive cultures onto the same YPS medium solidified with 1% (w/v) phytagel (Sigma) as a gelling agent and incubating in an anaerobic jar (Difco) prepared under a gas phase of N_2/CO_2 (80/20, 1 bar) at 80°C for 2 days. Cream-colored colonies were randomly picked, purified, and streaked on YPS-phytagel four times successively. Purified isolate was checked microscopically by a serial dilution step and designated NA1^T. The strain was stored at 4°C and could serve as an inoculum for at least 1 year. For long-term storage, pure cultures in the exponential growth phase were stored anaerobically in cryotubes at −80°C by adding 5% (v/v) dimethyl sulfoxide (Sigma) as a cryoprotectant.

For the measurement of cell growth, culture samples were diluted in sterile water containing NaCl (3%), formalin (2.5%), and 4'-6-diamidino-2-phenlyindole (0.01%), filtered with black polycarbonate membrane filters (pore size, 0.2 μm; Whatman), and counted with phase-contrast microscopy (Zeiss Axioplan) [21]. Optical density at 600 nm (OD_{600}) was also measured with a spectrophotometer (UV-2401PC, Shimadzu) for determination of optimum growth conditions. Before the measurements, serum vials were gently mixed by inversion, and sulfur particles were allowed to settle for 7 min. The correlation between turbidity of cultures in YPS medium and cell number was determined, and a regression analysis yielded the following formula: cells/ml=$9.2\times10^8\times OD_{600}$ (n=7, r^2=0.99). We used YPS medium unless otherwise stated.

To determine the optimal temperature for growth, cells were cultivated in serum vials, under N_2 gas (100%, 100 kPa) as headspace. To determine growth rates at different pHs, YPS medium was modified by using the following buffers (Sigma), each at a concentration of 20 mM: pH 3.5–4.5, no buffer; pH 5.0–6.0, 2-(*N*-morpholine)ethanesulfonic acid (MES); pH 6.5–7.0, piperazine-*N*,*N*'-bis2-ethanesulfonic acid (PIPES); pH 7.5–8.5, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES); and pH 9.0–10.0, no buffer. The optimal NaCl concentration for growth was determined by increasing the concentrations of NaCl added to the YPS medium. For all concentrations of NaCl, pH was adjusted to 8.5 prior to autoclaving. A constant temperature of 80°C was used while determining the optimal pH and NaCl concentration. Final anaerobiosis was achieved by adding 5% (w/v) sterile $Na_2S\cdot9H_2O$ to a final concentration of 0.025%. Each experiment was performed in duplicate.

The ability of isolate NA1^T to utilize different carbon sources for growth was tested at the optimal growth temperature on YPS medium (yeast extract and peptone were omitted) supplemented with 1% (v/v) filter-sterilized vitamin solution [4], with N_2 gas used as headspace. The medium was supplemented with one of the following carbon sources: starch, maltose, gelatin, glycogen, sucrose, cellobiose, xylose, and lactose, to a final concentration of 0.5% (w/v), whereas yeast extract, peptone, tryptone, casein, beef extract, casamino acids, succinate, propionate, pyruvate, and acetate were added to a final concentration of 0.2% (w/v). Possible autotrophic growth was tested in the basal medium using H_2/CO_2 (80:20, 200 kPa) headspace gas as the sole carbon source.

A mixture of 20 amino acids was tested for growth. A stock solution containing each amino acid at a concentration of 2.0 mM was sterilized separately by filtration (pore size, 0.22 μm; Whatman). The final amino acid mixture in the basal medium contained each of the 20 classical amino acids at a concentration of 0.2 mM. The requirement for elemental sulfur was determined by comparing the growth yield in YPS medium and in the same medium lacking elemental sulfur. Elemental sulfur was replaced with cystine (10%), polysulfide (10 mM), sodium thiosulfate (10 mM), sodium sulfate (20 mM), and sodium sulfite (3 mM) to investigate the ability of the isolate to use an alternative electron acceptor.

The morphology and mobility of strain NA1^T were observed under a phase-contrast microscope (Zeiss Axioplan) at 1,000× magnification. For scanning electron microscopy, cells were cultured at the end of the exponential growth phase and were then centrifuged. Cells were fixed for 2 h at 4°C in 2% glutaraldehyde in phosphate-buffered saline (PBS) buffer (pH 7.5), and then filtered with polycarbonate membrane filters (pore size, 0.22 μm; Whatman). After dehydration with increasing ethanol concentrations, samples were critical-point dried, coated with gold (SCD040, Balzers), and examined with a scanning electron microscope (JSM-840A; Jeol). For negative staining, 500 μl of bacterial suspension fixed with 2.5% glutaraldehyde (w/v) were dropped on Formvar/carbon-coated grids (300 mesh) and stained with 2% phosphotungstic acid (w/v). Micrographs were taken with a model JEM 1010 electron microscope (Jeol).

Cells of strain NA1^T were motile by means of polar flagella, coccoid-shaped, and occurred singly (Fig. 1). The size of the organisms ranged approximately from 0.5 to 1.0 μm in diameter. Colonies formed after 2 days of incubation on YPS at 80°C were circular, opaque, cream-colored, and convex with entire margins.

The strain was able to grow at temperatures ranging from 63 to 90°C and showed optimum growth at 80°C. No growth was detected at 60 or 95°C. Growth was supported within a pH range of 5.0 to 9.0, with an optimum pH of 8.5. No growth occurred at pH 4.5 or 9.5. The NaCl concentrations supporting growth were between 1.0 and 5.0% (w/v), with the optimal concentration at 3.5% (w/v). No growth was detected at the NaCl concentration of 0 or 5.5%

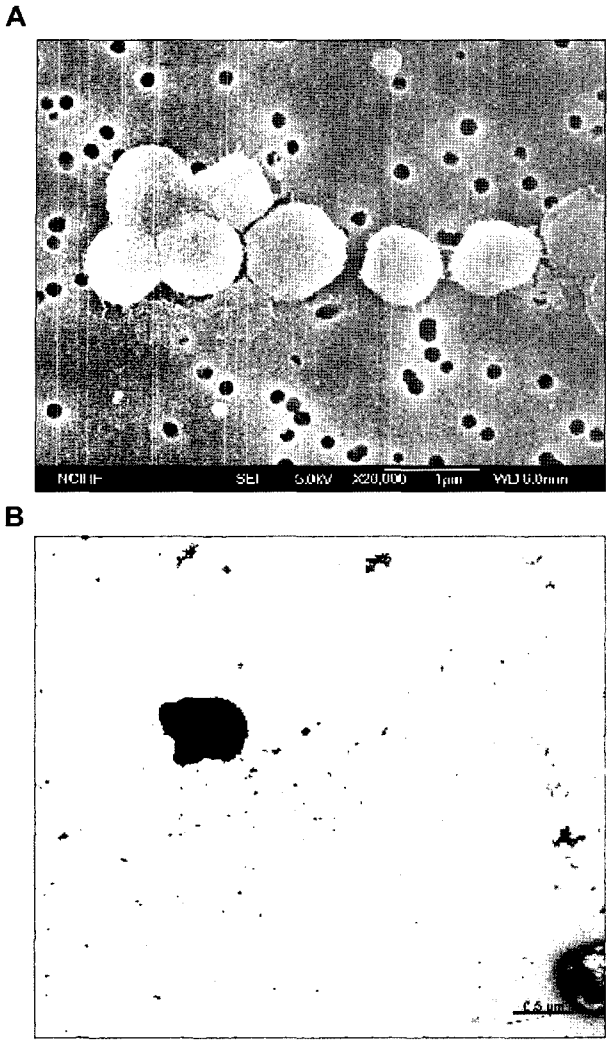

Fig. 1. Electron micrographs of strain NA1^T: **A**. Scanning electron micrograph; **B**. Transmission electron micrograph.
Scale bar represents 1 μm in **A** and 0.5 μm in **B**.

(w/v). The doubling time of strain NA1^T was approximately 92 min under optimal growth conditions (Fig. 2). Strain NA1^T appeared to be an obligate heterotroph. No growth was observed in mineral medium in the absence of a carbon source with an H_2/CO_2 gas headspace (80:20), with or without elemental sulfur. Elemental sulfur was necessary for growth and was reduced to hydrogen sulfide. No growth was detected in the presence of cystine, polysulfide, sodium thiosulfate, sodium sulfate, or sodium sulfite as an electron acceptor. Significant growth was observed on beef extract, casein, peptone, tryptone, yeast extract, and starch. Strain NA1^T was also able to grow on a mixture of 20 amino acids. No growth was observed on maltose, lactose, sucrose, cellobiose, xylose, gelatin, glycogen, casamino acids, acetate, succinate, propionate, and pyruvate. Differential physiological characteristics of strain NA1^T with related hyperthermophilic *Thermococcus* strains are summarized in Table 1.

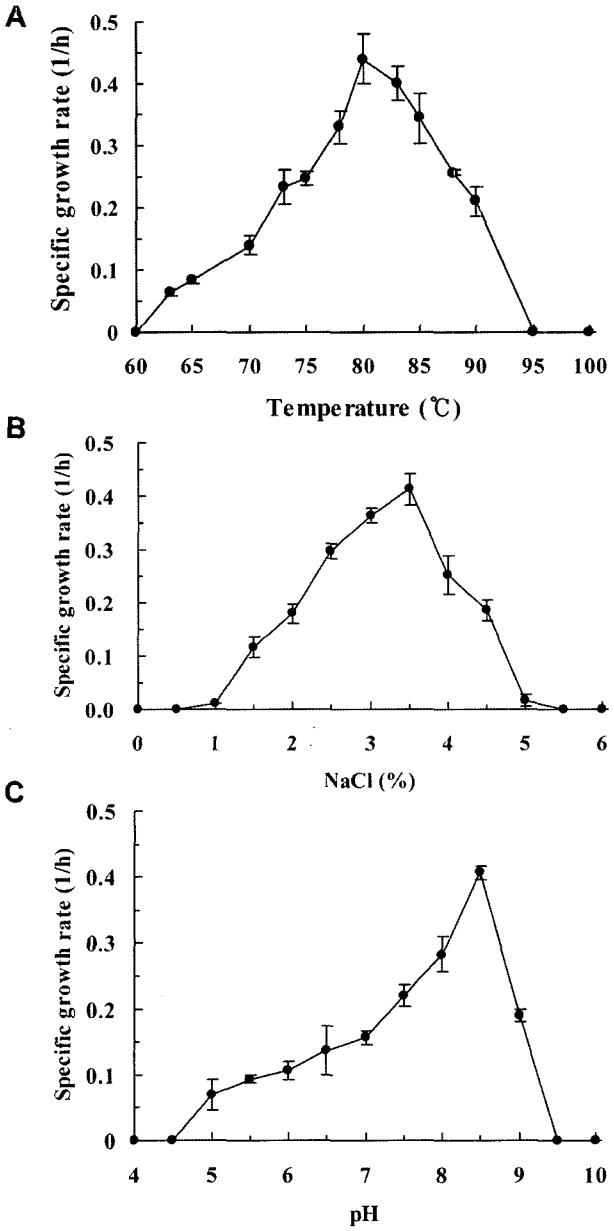

Fig. 2. Effects of temperature, (**A**) NaCl (**B**), and pH (**C**) on growth of strain NA1^T.

Genomic DNA was prepared following the method of Godfroy *et al.* [12]. The 16S rDNA gene was prepared and purified as described by Sohn *et al.* [26] using two specific archaeal primers, Arch21F and Arch1492R [7]. PCR products were cloned in pGEM T-easy vector (Promega) and sequenced using a BigDye terminator cycle sequencing kit (PE Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The central portion of the 16S rDNA fragments was sequenced using the primers Arch533F (5'-GTGCCAGCAGCCGCGGTAA-3') and Arch958R (5'-YCCGGCGTTGAMTCCATTT-3'). The nucleotide sequence has been submitted to the GenBank/

Table 1. Physiological characteristics of strain NA1^T and its phylogenetic relatives.

Property	1	2	3	4	5
Growth substrate					
Casein	+	ND	+	ND	+
Amino acids	+	-	ND	+	-
Starch	+	-	NR	+	-
Maltose	-	-	NR	+	-
Pyruvate	-	W	+	+	-
Sulfur requirement	R	R	S	R	S
Growth temperature (°C)					
Range	63–90	68–95	93	60–100	60–100
Optimum	80	80–88	88	85	85
NaCl concentration (%)					
Range	1–5	1–5	ND	1–5	1–5
Optimum	3.5	2–3.5	4	3	3
pH					
Range	5–9	5.8–8.5	NR	5–9	3–8
Optimum	8.5	6.5–7.2	5.8	6.5	6
G+C content (mol%)	52	50.6	56.6	52	52

Strains: 1, NA1^T; 2, *T. gorgonarius* [19]; 3, *T. celer* [33]; 4, *T. kodakaraensis* [1]; 5, *T. peptonophilus* [13]. +, positive; -, negative; W, weak reaction; ND, not determined; NR, not reported; R, required; S, stimulatory.

EMBL Data Bank with Accession No. DQ167232. Phylogenetic analysis of the 16S rDNA gene sequence was performed as previously described by Bae *et al.* [2]. Phylogenetic trees were inferred using the Fitch-Margoliash [10], maximum-parsimony [11], and neighbor-joining [23] algorithms. Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated according to the model of Jukes and Cantor [15]. The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analyses [8] of the neighbor-joining method based on 1,000 resamplings. The 16S rDNA sequence of strain NA1^T was a continuous stretch of 1,457 bp. Sequence analysis of this isolate revealed that it belonged to the genus *Thermococcus* (Fig. 3). Based on 16S rRNA gene sequence similarities, the closest relatives of strain NA1^T are *T. gorgonarius* (99.7%), *T. kodakaraensis* (99.5%), *T. peptonophilus* (99.5%), and *T. celer* (99.4%).

Taking the phylogenetic tree based on 16S rDNA gene sequence into consideration, we performed DNA-DNA hybridization of strain NA1^T with the four phylogenetically related type strains of genus *Thermococcus*. DNA-DNA hybridization was performed according to the method of De Ley *et al.* [6]. When strain NA1^T was used as the labeled

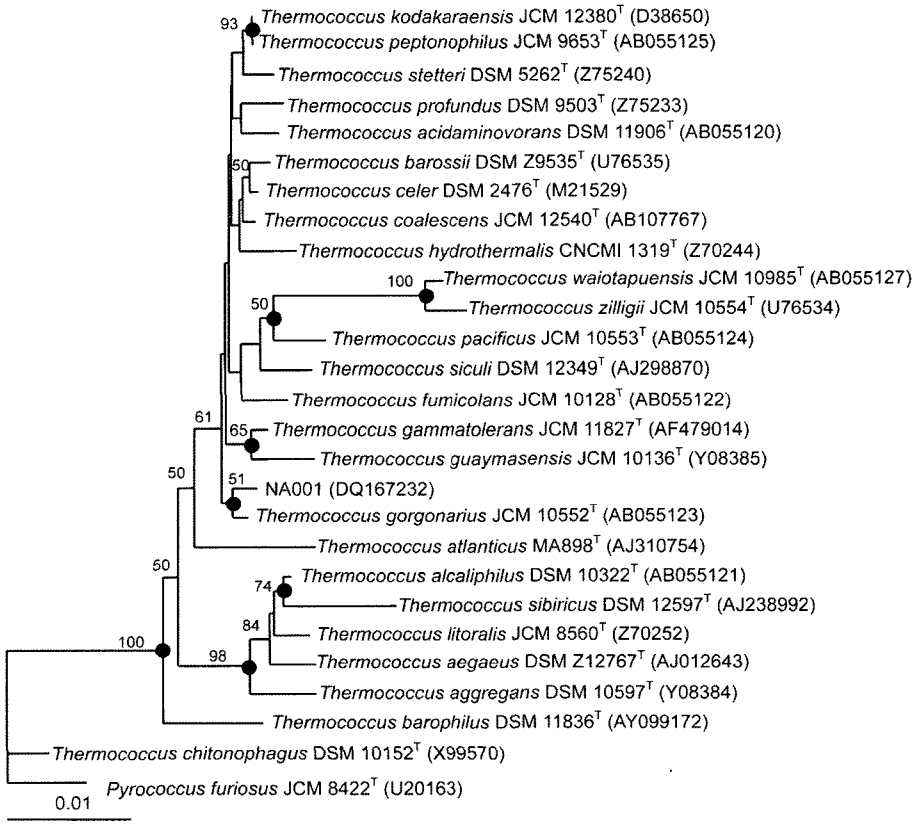

Fig. 3. Neighbor-joining tree based on nearly complete 16S rDNA sequences, showing relationships between strain NA1^T and related taxa of the genus *Thermococcus*.

Bootstrap percentage values higher than 50% based on neighbor-joining analyses of 1,000 replications were included at the nodes, and solid circles indicate that corresponding nodes (groupings) were also recovered in Fitch-Margoliash and maximum-parsimony. Bar, 0.01 nucleotide substitution per position.

probe, the levels of DNA-DNA hybridization were as follows: 26.4%, *T. peptonophilus*; 24.0%, *T. gorgonarius*; 15.5%, *T. celer*; 6.8%, *T. kodakaraensis*. G+C content (mol%) of the isolate NA1^T was determined by the melting temperature method, as described by Mandel *et al.* [17] and Marmur and Doty [18], with the melting temperature of purified chromosomal DNA from *Escherichia coli* K-12 (KCTC 2443) also determined to serve as a control. The G+C content of strain NA1^T was 52.0 mol%.

On the basis of the 16S rDNA sequence comparison, the hyperthermophilic strain NA1^T belongs to the genus *Thermococcus*. The 16S rDNA sequence data were useful for assigning the isolate NA1^T to the genus level, but the similarities to other species within the genus were too high to allow differentiation at the species level. Species of *Thermococcus* showing higher than 99.4% 16S rDNA sequence similarity to the isolate NA1^T were therefore compared by DNA-DNA hybridization. The results clearly showed that the hybridization values were lower than 70%, indicating that strain NA1^T represents a novel species [27, 31]. Furthermore, *T. gorgonarius*, which is the nearest phylogenetic relative of isolate NA1^T, possesses a morphologically characteristic protrusion (prostheca-like structure) that is absent in strain NA1^T, and unlike strain NA1^T, it does not grow in amino acids and starch [19]. The optimal pH of *T. gorgonarius* is also much lower (pH 6.5–7.2) than that of strain NA1^T (pH 8.5). Moreover, strain NA1^T differs in optimal temperature and pH for growth with its closest phylogenetic relatives (Table 1). On the basis of its physiological and genetic characteristics, strain NA1^T represents a novel species within the genus *Thermococcus*.

Description of *Thermococcus onnurineus* sp. nov.

Thermococcus onnurineus (*on.nu.ri'ne.us.* N.L. masc. adj. *onnurineus,* derived from *R/V Onnuri*, the vessel during the "DAE-YANG" cruise). Cells are coccoid-shaped (0.5–1.0 μm in diameter), motile by means of polar flagella, and singular. Colonies formed after 2 days at 80°C are circular, opaque, and cream-colored. The cells are obligately anaerobic. Growth occurs at 63–90°C, with the optimum at 80°C. This species grows optimally at pH 8.5 and in the presence of 3.5% NaCl, and it is obligately heterotrophic. It grows preferentially on proteolysis products such as beef extract, casein, peptone, tryptone, and yeast extract, and on starch as the carbon source. This species does not grow on maltose, lactose, sucrose, cellobiose, xylose, gelatin, glycogen, casamino acids, acetate, succinate, propionate, or pyruvate. Elemental sulfur is necessary for growth. Cystine, polysulfide, sodium thiosulfate, sodium sulfate, and sodium sulfite are not used as electron acceptors. DNA G+C content is 52.0 mol%. The hyperthermophilic strain NA1^T was isolated from deep-sea sediment within the PACMANUS field (1,650 m in depth). The type strain is NA1^T (=KCTC 10859^T, =JCM 13517^T).

Acknowledgments

We thank the chief scientist of the "DAE-YANG" cruise (2001), Dr. S. M. Lee, and the captain and crew of the *R/V Onnuri*. We also thank Professor J. P. Euzéby for the nomenclature. This work was supported by the Extreme Molecular Genomics Research Program of the Ministry of Marine Affairs and Fisheries and the In-house Project (PE97201) of the Korea Ocean Research and Development Institute (KORDI), Republic of Korea.

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