Vol. 27(2):215-219 | Ocean and Polar Research | June 2005

Article

Isolation of Protease-Producing Arctic Marine Bacteria

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Abstract : We isolated and identified three protease-producing bacteria that had inhabited the region around the Korean Arctic Research Station Dasan located at Ny-Alesund, Svalbard, Norway (79°N, 12°E). Biofilms were collected from the surface of a floating pier and from dead brown algae in a tide pool near the seashore. The biofilm samples were transported to the Korea Polar Research Institute (KOPRI) under frozen conditions, diluted in sterilized seawater, and cultured on Zobell agar plates with 1% skim milk at 10°C. Three clear zone forming colonies were selected as protease-producing bacteria. Phylogenetic analysis based on 16S rDNA sequences showed that these three stains shared high sequence similarities with *Pseudoalteromonas elyakovii*, *Exiguobacterium oxidotolerum* and *Pseudomonas jessenii*, respectively. We expect these Arctic bacteria may be used to develop new varieties of protease that are active at low temperatures.

Key words : Arctic, Marine bacteria, Protease, *Exiguobacterium*, *Pseudoalteromonas*, *Pseudomonas*

1. Introduction

Proteases, the enzymes cutting long sequences of amino acids into protein fragments, are essential for all life forms. Proteases are necessary for the synthesis of proteins, controlling protein composition, size, shape, turnover and ultimate destruction. Their actions are exquisitely selective; each protease splits very specific sequences of amino acids. Therefore, there are many different types of proteases; e.g. humans have over 5,000 different protease genes, accounting for 2% of total human genes (Puente *et al.* 2003).

Proteases show potential commercial applications. They are important enzymes in the medical, environmental, food, and chemical industries, etc. (Storer 1991; James and Simpson 1996; Vermeij and Blok 1996).

Proteases have been isolated from several marine bacteria (Alfredsson *et al.* 1995; Marcello *et al.* 1996; Irwin *et al.* 2001; Lee *et al.* 2002; Arnorsdottir 2005). These bacterial proteases showed algicidal activity or enhanced chitinase activity (Lee *et al.* 2002; Miyamoto *et al.* 2002). There have been a few reports on protease-producing Arctic bacteria (Irwin *et al.* 2001; Lee *et al.* 2004). The protease released by Arctic bacteria showed optimal activity at low temperatures (Huston *et al.* 2000). Therefore, the Arctic region can be a good source offering cold-active enzymes.

In this study, we isolated and identified protease-producing marine bacteria that have inhabited the marine environment around Korean Arctic Research Station, Dasan.

2. Materials and methods

Sample collection

Biofilm samples were collected from the region around the Korean Arctic Research Station Dasan located at Ny-Alesund, Svalbard, Norway (79°N, 12°E) on the 2nd August 2003 (Fig. 1). Biofilms were collected from the surface of a floating pier and from dead brown algae in a tide pool near the seashore using a sterilized razor, which were then placed in new 50 m*l* conical tubes. The collected biofilm samples were frozen in a −20°C freezer, and transported to

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Fig. 1. Sampling sites in this study. (a) A floating pier around the Korean Arctic Research Station Dasan located at Ny-Alesund, Svalbard, Norway, (b) biofilms formed on the floating pier, (c) a tide pool near the seashore, and (d) dead brown algae in the tide pool.

the laboratory of the Korea Polar Research Institute (KOPRI) under freezing conditions in a Biocooler (Biosmith, USA). The samples were diluted 50 to 1000-fold in sterilized seawater and spread on Zobell agar plates fortified with 1% skim milk (skim milk 10 g, peptone 5 g, yeast extract 1 g, $FePO_4$ 0.01 g, agar 15 g, sterilized water 250 m*l*, aged sea water 750 m*l*, pH 7.0). Following a seven-day incubation period at 10°C, colonies forming clear zones were selected as protease-producing bacteria.

DNA extraction and PCR amplification

Total genomic DNA was extracted using an AccuPrep genomic DNA extraction kit (Bioneer, Korea) from 1 m*l* of isolates cultured in Zobell broth. From the genomic DNA nearly full-length 16S rDNA sequences were amplified by PCR using primers 27F (5'-AGA GTT TGA TCN TGG CTC AG-3') and 1522R (5'-AAG GAG GTT ATC CAN CCR CA-3'). The PCR mixture consisted of 5 μl of 10× PCR mixture (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μl of each primer, 1 μl of template DNA, and 2.5 units of *Taq* polymerase (TaKaRa, Japan) in a final volume of 50 μl. The PCR was performed in a thermal cycler (Biometra, Germany) using cycling conditions that consisted of an initial denaturation at 95°C for 5 min and then 30 cycles with denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. A final extension was performed at 72°C for 7 minutes. The PCR products were analyzed by agarose gel electrophoresis, and purified with an AccuPrep PCR purification kit (Bioneer, Korea).

Sequence analysis

Full-length sequences of amplified 16S rDNA genes were obtained using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). The nucleotide sequences were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Sequences of the 16S rDNA were submitted to the Advanced BLAST search program to identify sequences of closely related organisms. The related sequences were preliminarily aligned with CLUSTALW (Thompson *et al.* 1994), and complete sequence alignments were performed using PHYDIT (Chun 1995) and manual comparison. Phylogenetic analyses were performed with PHYLIP (Felsenstein 1993), and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei 1987).

3. Results and discussion

We isolated three strains designated KOPRI 20234, KOPRI 20246, and KOPRI 20255. They formed a clear zone around the colonies on Zobell agar media containing

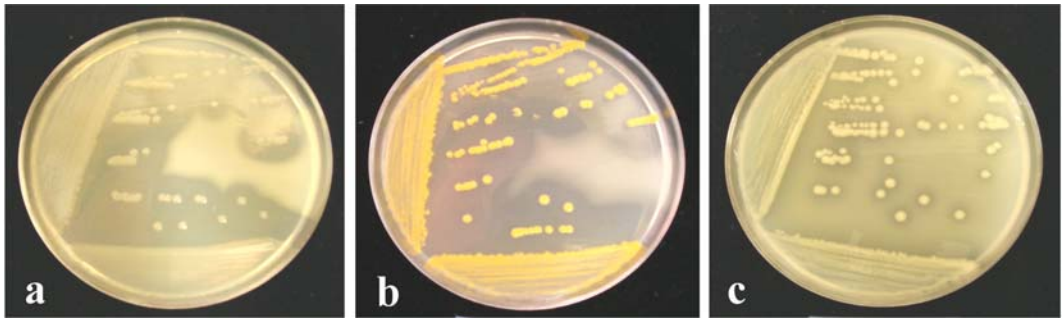

Fig. 2. Arctic marine bacteria showing protease activity. (a) KOPRI 20234, (b) KOPRI 20246, and (c) KOPRI 20255.

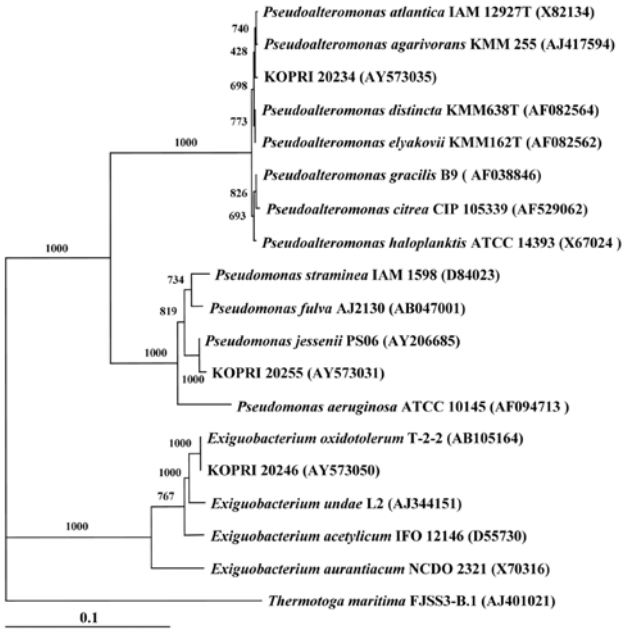

Fig. 3. Neighbor-joining tree of 16S rDNA sequences of three Arctic marine bacteria showing protease activity. The sequences were aligned and used to construct a neighbor-joining tree based on the Kimura-2 parameter. The scale bar indicates the branch length that corresponds to 0.1 substitutions per position.

skim milk (Fig. 2). Phylogenetic analysis using 16S rDNA indicated that strains KOPRI 20234, KOPRI 20246, and KOPRI 20255 shared high similarities with *Pseudoalteromonas elyakovii*, *Exiguobacterium oxidotolerum* and *Pseudomonas jessenii*, respectively (Fig. 3). Because they could grow over 25°C, they may be not psychrophilic but psychrotolerant species.

Pseudoalteromonas sp. KOPRI 20234

Strain KOPRI 20234 was isolated from the biofilm formed on the surface of a floating pier in a small harbor. The phylogenetic analysis indicated that strain KOPRI 20234 belongs to the representative marine bacterial genus *Pseudoalteromonas.* It showed a high 16S rDNA sequence similarity with *P. atlantica*, *P. distincta, P. elyakovii* (99.72%), and with *P. agarivorans* (99.58%). Even though strain KOPRI 20234 shares a high sequence similarity with these four species, it does not show agarolytic ability, which is the key characteristic of *P. agarivorans* and *P. atlantica* (Akagawa-Matsushita *et al.* 1992; Romanenko *et al.* 2003). It also does not produce the brown pigment that is observed in *P. atlantica* and *P. distincta*. (Romanenko *et al.* 1995; Sawabe *et al.* 2000). Because *P. atlantica*, *P. distincta* and *P. elyakovii* share over 99.72% sequence similarities with one another, KOPRI 20234 could not be assigned to a single *Pseudoalteromonas* species by the phylogenetic analysis alone. Therefore, DNA-DNA hybridization analysis is necessary to clarify the taxonomic relationship between strain KOPRI 20234 and the four *Pseudoaleromonas* species.

Exiguobacterium oxidotolerans KOPRI 20246

Strain KOPRI 20246 was isolated from the surface of a dead brown alga collected in a tide pool near the seashore. Strain KOPRI 20246 showed 100% 16S rDNA sequence similarity with *Exiguobacterium oxidotolerans*. Strain KOPRI 20246 was orange in color, which is the color of the type strain of *E. oxidotolerans* (Yumoto *et al.* 2004). Type strains of two *Exiguobacterium* species have been isolated from a microbial mat from Lake Fryxell, Antarctica (Fruhling *et al.* 2002). Two new species have been reported from the marine environment such as the tidal flat (Kim *et al.* 2005). This is the first report on Arctic marine *Exiguobacterium* species producing protease.

Pseudomonas jessenii KOPRI 20255

Strain KOPRI 20255 was isolated from the surface of a dead brown alga collected in a tide pool near the seashore.

The colonies of KOPRI 20255 were ivory in color. Strain KOPRI 20255 shared 99.59% 16S rDNA sequence similarity with *Pseudomonas jessenii* (Verhille *et al.* 1999), of which the type strain was isolated from natural mineral waters.

Further studies are needed to elucidate specific activity and optimal conditions for these Arctic bacterial proteases. We expect these Arctic bacteria can be used for screening new enzymes that are active at low temperatures.

Acknowledgements

We thank the reviewers for their critical and useful comments. This work was supported by a grant PE05006 from Korea Polar Research Institute.

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Received May 2, 2005
Accepted May 20, 2005