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Diversity of proteolytic microbes isolated from Antarctic freshwater lakes and characteristics of their cold-active proteases

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ABSTRACT

Despite being an extreme environment, the water temperature of freshwater lakes in Antarctica reaches 10 °C in summer, accelerating biological activity. In these environments, proteolytic microbial decomposers may play a large role in protein hydrolysis. We isolated 71 microbial strains showing proteolytic activity at 4 °C from three Antarctic freshwater lakes. They were classified as bacteria (63 isolates) and eukaryotes (8 isolates). The bacterial isolates were classified into the genera *Flavobacterium* (28 isolates), *Pseudomonas* (14 isolates), *Arthrobacter* (10 isolates), *Psychrobacter* (7 isolates), *Cryobacterium* (2 isolates), *Hymenobacter* (1 isolate), and *Polaromonas* (1 isolate). Five isolates of *Flavobacterium* and one of *Hymenobacter* seemed to belong to novel species. All eukaryotic isolates belonged to *Glaciozyma antarctica*, a psychrophilic yeast species originally isolated from the Weddell Sea near the Joinville Island, Antarctica. A half of representative strains were psychrophilic and did not grow at temperatures above 25 °C. The protease secreted by *Pseudomonas prosekii* strain ANS4-1 showed the highest activity among all proteases from representative isolates. The results of inhibitor tests indicated that nearly all the isolates secreted metalloproteases. Proteases from four representative isolates retained more than 30% maximal activity at 0 °C. These results expand our knowledge about microbial protein degradation in Antarctic freshwater lakes.

1. Introduction

Antarctica is one of the most extreme environments for living creatures owing to various factors such as a cold temperature, aridity, strong wind, and ultraviolet radiation. However, ice-free areas, which exist in coastal regions and account for only 2% of the Antarctic continent, are oases for creatures (Kimura et al., 2010; Michaud et al., 2012). In particular, in Antarctic lakes located in ice-free areas, the water temperature increases up to 10 °C in summer (Imura and Kanda, 2002); therefore, biological activity is relatively high in Antarctic environments. This biota is very simple because of the absence of organisms that generally occupy the top of the food webs (Laybourn-Parry, 2002; Tanabe et al., 2017). However, there are mat-like plant communities composed of algae and mosses, as well as animalcules such as rotifers, nematodes, and water bears (Tardigrades), with biomasses that are extremely rich compared with that of terrestrial organisms (Imura and Kanda, 2002; Tsujimoto et al., 2014). Cold-adapted microbes, which are at the bottom of this simple food chain, produce cold-active enzymes as a survival strategy at low temperatures (Singh et al., 2014). These enzymes contribute to the circulation of organic matter in the cold environment (Vazquez et al., 2004). Among the enzymes, cold-active proteases may play an important role in hydrolyzing proteins, which are one of the major high-molecular-weight compounds produced by all living organisms in this environment. On the other hand, because of their potential for various industrial applications, such as detergent and food processing at low temperatures, cold-active proteases also attract the attention of many researchers (Brenchley, 1996; Joshi and Satyanarayana, 2013). Some

Abbreviations: DIN, dissolved inorganic nitrogen; LB, Luria–Bertani; MBS, Modified Brock's basal salt; MBSY, MBS with 0.1% yeast extract; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; SSU rRNA, small-subunit ribosomal RNA.

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cold-active proteases secreted by microbes from Antarctica have been characterized (Ray et al., 1992; Villeret et al., 1997; Narinx et al., 1997; Dube et al., 2001; Turkiewicz et al., 2003; Wang et al., 2005; Lario et al., 2015; Santos et al., 2015). However, these studies were conducted on one or a small number of microbial species, and thus, the diversity of Antarctic proteolytic microbes had not been elucidated. Although Vazquez et al. (1995), Zhou et al. (2013), and Shivaji et al. (2010) described many isolates of Antarctic proteolytic bacteria, they did not investigate the optimal temperature of each protease. To understand the microbial degradation of proteins in Antarctica, varying temperature effects on proteases must be elucidated as well as many isolates must be identified.

In this study, we isolated proteolytic microbes from three Antarctic freshwater lakes located in ice-free areas of the coastal region of Lützow–Holm Bay, East Antarctica, and examined their diversity. We also classified the proteases secreted by the isolates and investigated their temperature characteristics.

2. Materials and methods

2.1. Sampling

Water samples, including surface sediments, were collected from three lakes—Lake Yukidori Ike (69°14′26″S, 39°45′23″E) in the Langhovde area, Lake Hotoke Ike (69°28′36″S, 39°33′41″E) in the Skarvsnes area, and Lake Skallen Oike (69°40′22″S, 39°24′39″E) in the Skallen area located in the coastal region of Lützow-Holm Bay, East Antarctica—in December 2012 through January 2013 during the 54th Japanese Antarctic Research Expedition. Water temperatures and pH of the samples were recorded at each sampling site and are shown in Table 1. The samples were taken from three places at each lakeshore using sterile stainless spoon. The samples from each lake were mixed and stored at −30 °C and at 4 °C.

2.2. Isolation of proteolytic microbes

The frozen and chilled samples from each lake (n = 6) were used in this study. The frozen samples were slowly thawed at room temperature before cultivation. A volume of 0.1 mL of each water sample, including surface sediments, was spread on Luria–Bertani (LB) agar plates supplemented with 30 g/L skim milk and on modified Brock's basal salt (MBS) (Kurosawa et al., 1998) agar plates supplemented with 0.1% yeast extract and 30 g/L skim milk. The plates were incubated at 4 °C for 10–55 days. Colonies with a halo (clear zone formed upon degradation of skim milk by a protease), indicating proteolytic activity, were randomly selected and purified by repeated single-colony isolation on the same medium at 4 °C for 23–62 days. The isolates were then incubated in 10 mL of LB or MBS with 0.1% yeast extract (MBSY) liquid medium at 4 °C for 7–42 days. A 7-mL aliquot of a pure-culture suspension was centrifuged, and the supernatant was removed, while the cell pellet was stored at −25 °C for DNA extraction. Another 3 mL of the pure-culture suspension was dispensed into 1.5-mL Eppendorf tubes with 10% dimethyl sulfoxide and stored at −80 °C for further experiments.

2.3. Identification of isolates and phylogenetic analysis

The cell pellet containing approximately 8×10^7–7×10^8 cells of each strain was suspended in 200 μL of TE buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0) containing 0.2% Triton X-100 and heated at 75 °C for 5 min. DNA was extracted using a DNA extraction machine (Magtration system 12GC, Precision System Science Co., Ltd., Matsudo, Japan) with the MagDEA DNA 200 DNA extraction reagent (Precision System Science Co., Ltd., Matsudo, Japan). Partial small-subunit ribosomal RNA (SSU rRNA) gene sequences were amplified by polymerase chain reaction (PCR) using universal bacteria-specific primers (B27F and U1492RM) and eukaryote-specific primers (EK82F and EK1592R). The PCR conditions for bacterial 16S rRNA gene amplification were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. For eukaryotic 18S rRNA gene amplification, the PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 2 min, followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The partial SSU rRNA gene fragments of the isolates were sequenced, and the sequences were compared with those of published species in the DDBJ/EMBL/GenBank databases using EzTaxon (http://www.ezbiocloud.net/eztaxon) and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). To analyze the phylogenetic relationships between the isolates and published species, neighbor-joining trees, including bootstrap probabilities (1000 samplings), were constructed using the GENETYX version 11.0.1 and MEGA6 software.

2.4. Evaluation of protease activity and growth of isolates

To evaluate the proteolytic activity and growth of each isolate on an agar plate, 1 μL of a culture was inoculated on MBSY agar plates supplemented with 30 g/L skim milk, and the plates were incubated at 4 °C and 25 °C for 14–35 days. The growth of each strain was evaluated based on colony formation. The protease activity was evaluated based on the ratio of the area of the halo to that of the colony.

2.5. Protease assay

The assay of the protease activity was performed using a modified method of Park et al. (2014). Representative isolates were cultured in 5 mL of MBSY liquid medium supplemented with 30 g/L skim milk at 4 °C for 13–34 days depending on the growth rate of

Table 1
Sampling sites and numbers of isolates.

Sampling site	Water temperature (°C)	Water pH	Number of isolates on different media and from samples stored at different temperatures				
			LB agar plate		MBSY agar plate		Total
			−30 °C	4 °C	−30 °C	4 °C	
Lake Yukidori Ike	5.4	8.4	2	2	7	9	20
Lake Hotoke Ike	5.5	7.9	4	14	3	3	24
Lake Skallen Oike	6.1	7.8	10	4	10	3	27
Total			16	20	20	15	71

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