• Article •

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Cold-adaptive alkaline protease from the psychrophilic *Planomicrobium* sp. 547: enzyme characterization and gene cloning

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Abstract A psychrophilic bacterium strain 547 producing cold-adaptive alkaline protease was isolated from the deep sea sediment of Prydz Bay, Antarctica. The organism was identified as a *Planomicrobium* species by 16S rRNA analysis. The optimal and highest growth temperatures for strain 547 were 15℃ and 30℃, respectively. The extracellular protease was purified by ammonium sulfate precipitation and DEAE cellulose-52 chromatography. The optimal temperature and pH for the activity of the purified enzyme were 35℃ and pH 9.0, respectively. The enzyme retained approximately 40% of its activity after 2 h of incubation at 50℃. The enzymatic activity was inhibited by 1 mmol/L phenylmethyl sulfonylfluoride (PMSF) and hydrochloride 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), indicating that it was a serine protease. The presence of Ca^{2+} and Mn^{2+} increased the activity of the enzyme. The protease gene with a size of 1 269 bp was cloned from *Planomicrobium* sp. 547 using primers designed based on the conserved sequences of proteases in GenBank. The *Planomicrobium* sp. 547 protease contained a domain belonging to the peptidase S8 family, which has a length of 309 amino acid (AA) residues. The alignment and phylogenetic analysis of the AA sequence indicated that the protease belonged to the subtilisin family.

Keywords **cold-adaptive protease, *Planomicrobium*, Antarctic, subtilisin**

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0 Introduction

Proteases act as processing enzymes that participate in regulatory or catabolic processes in the cell or as extracellular enzymes that play an important role in the degradation of proteinaceous substrates that serve as carbon or energy sources[1]. As the largest superfamily of proteases, the subtilisin-like proteases, which are also known as subtilases, have been widely studied. There are many reports describing subtilases such as protease from alkaliphilic *Bacillus* sp. no. 221[2], Maxacal™ from *Bacillus alcalophilus* PB92[3], Savinase™ from *Bacillus lentus*[4], and an oxidatively stable alkaline serine protease from *Bacillus* KP-43[5].*alcalophilus* PB92[3], Savinase™ from serine protease from *Bacillus* KP-43[5]. In addition to enzymes from bacteria, subtilases from fungi[6–7] and archaea[8] have also been receiving increased attention. Furthermore, subtilases have been used in various industrial applications, such as detergents, food and feed production, and leather and textile processing[9]. Although many proteases have been studied, the discovery of new proteases is still significant for both commerce and research. This is especially true for proteases from extreme environments such as the deep sea and Antarctica, because of their novel characteristics. Here, we report the screening, characterization and gene cloning of a cold-adaptive alkaline protease from a psychrophilic bacterium strain isolated from deep sea sediment of Prydz Bay, Antarctica.

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1 Materials and methods

1.1 Screening and identification of strain

Strain 547, which showed strong caseinolytic activity, was isolated from deep sea sediment from Prydz Bay, Antarctica (74º25′E, 66º55′S, 900 m). The sediments were diluted with sterile water and the supernatants were grown at 10℃ on screening medium (pH 10.0), which contained 2 g yeast extract, 10 g casein and 15 g agar per liter of seawater. The identification was conducted by 16S rRNA analysis. Genomic DNA from strain 547 was prepared using a Genome Extraction Kit (Bioteke, China). The primers for the PCR reaction were universal bacteria primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-AAGGAGGTGATCCAGCC-3'). The amplification was conducted by subjecting the samples to an initial denaturation step of 4 min at 95℃ and then 30 cycles of 1.5 min of denaturation at 94℃, annealing at 55℃ for 1.5 min, and 1.5 min at 72℃ for extension. The final step consisted of 10 min at 72℃ and storage at 4℃. The amplified 16S rRNA was cloned into pTA2 (Toyobo) and sequenced by Sangon Biotech Company (China).

1.2 Preparation of protease

The culture was grown on liquid screening medium as described above at 20℃ for four days. Cells and insoluble material were removed by centrifugation at 13 000 r/min for 10 min at 4℃ and the cell-free supernatant was then used as the crude enzyme preparation. All subsequent purification steps were conducted at 4℃. The crude extract was subjected to a two-step ammonium sulfate precipitation, and the protein precipitating at a salt saturation between 30% and 80% was resuspended in 10 mmol/L Tris-HCl buffer (pH 9.0). The sample was then dialyzed against the same buffer for 12 h. Next, the enzymes were subjected to anion exchange chromatography on a DEAE-52 column, after which they were eluted in a linear gradient between 0 and 0.5 M NaCl with 10 mmol/L Tris-HCl buffer (pH 9.0) containing 10 mmol/L $CaCl_2$. Finally, the fractions containing proteolytic activity were desalted and concentrated by dialysis and lyophilization, after which they were used for characterization.

1.3 Characterization of protease

The protease activity was determined using Folin & Ciocalteu's phenol reagent with casein serving as the substrate under the following conditions. The reaction mixture including 200 μL purified protease and 800 μL 2% (w/v) casein in 50 mmol/L Tris-HCl (pH 9.0) was incubated at different temperatures for 20 min and the reaction was stopped by adding 270 μL 10% (v/v) trichloroacetic acid. The precipitate was then removed by centrifugation at 12 000 r/min for 2 min. Next, 1 mL of supernatant was used to determine the amount of tyrosine. To accomplish this, 5 mL 0.4 mol/L Na_2CO_3 and 0.5 mL 1N Folin-Ciocalteu's Phenol reagent (Sigma) were added, and the sample was measured fluorometrically at 680nm. One unit of activity (U) was defined as the amount of the enzyme releasing 1μg of tyrosine per minute under the conditions described above. All assays were conducted in triplicate.

The effects of temperature on protease were determined by measuring the activity at 10–60℃ at an interval of 5℃. The thermo-stability of the protease was examined by measuring the residual activities of the enzyme fractions, which were pre-incubated at 50℃ in 0.1 mol/L Tris-HCl (pH 9.0) for 10 min, 20 min, 30 min, 40 min, 50 min, 60 min and 120 min. The optimum pH for the protease activity was determined at 35℃ by measuring the activity at pH 5–12. All pH values were adjusted at room temperature. The effects of metal ions and denaturing agents on protease were examined by determining the activities of the enzyme after 60 min of incubation at 20℃ in 0.1M Tris-HCl (pH9.0) buffers containing various metal ions (final concentration 1 mmol/L) and denaturing agents. The metal ions for determination included Ca^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Li^{2+}, Mg^{2+}, Na^{+}, K^{+}, Ni^{2+}, Fe^{2+} and Zn^{2+}.

1.4 Cloning of the protease gene

The following pair of primers was designed based on the conserved sequences of subtilases in GenBank: K1, 5'-AATGGNCATGGNACNCATGTNGCNGG-3' and K2, 5'-CGTGNGGNGCNGCCATNCTNGTNCC-3' (N: A or G or C or T). The PCR product with a size of 564 bp was applied to identify homologous sequences in GenBank. In addition, a second pair of primers was designed based on the sequences showing the highest homology with the 564 bp product as follows: PF, 5'-ATGAAGAGATCAGG-AAAGATTTTCA-3' and PR, 5' -TTATTGAACTGTTGC-AAATCCGAAC-3'. The amplification of primer pair PF and PR was conducted by subjecting the sample to an initial denaturation step of 4 min at 95℃ followed by 30 cycles of 1 min denaturation at 94℃, 1 min at 55℃ for annealing, and 1 min at 72℃ for extension using *ExTaq* polymerase (Takara). The final step consisted of 10 min at 72℃, after which the sample was stored at 4℃. The amplified protease gene was cloned into pTA2 (Toyobo) and then sequenced by Sangon Biotech Company (China).

1.5 Analysis of sequences

A BLAST search for homologous sequences of 16S rRNA and protease gene sequences was conducted (http://blast.

ncbi.nlm.nih.gov/Blast.cgi). Identified 16S rRNA and AA sequences were then aligned and subjected to phylogenetic analysis using DNAMAN and Clustal X program, respectively.

2 Results

2.1 Identification of *Planomicrobium* sp. 547

The alignment of the 16S rRNA sequence indicated that strain 547 was most closely related to the genera *Planococcus* and *Planomicrobium*, which both belong to Planococcaceae with more than 99% similarity. Phylogenetic analysis based on the 16S rRNA sequence indicated that strain 547 showed the highest homology with *Planomicrobium okeanokoites*[10]. The bacteria located in the neighboring clades on the phylogenetic tree all belonged to the genus *Planomicrobium*. Although the 16S rRNA sequences of the *Planococcus* strains showed high similarity to that of strain 547, they were located in different clades on the phylogenetic tree. Therefore, we placed strain 547 in the genus *Planomicrobium*, as *Planomicrobium* sp. 547 (Figure 1).

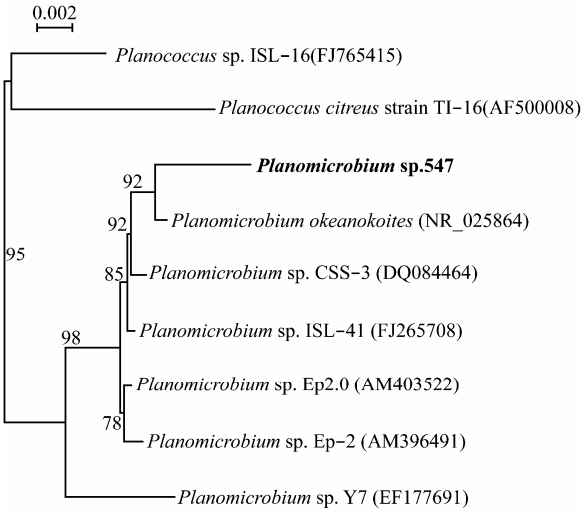

Figure 1 Phylogenetic analysis of *Planomicrobium* sp. 547 based on the 16S rRNA sequence.

2.2 Growth and protease-producing characteristics of *Planomicrobium* sp. 547

Planomicrobium sp. 547 produced a clear zone on the screening medium plate after culture at 10℃. The organism grew well at 4℃–20℃, but did not grow at temperatures higher than 30℃. Based on the density and stability of cell growth, 15℃ was the optimal temperature for growth of the organism. The carbon and nitrogen substrates utilized by *Planomicrobium* sp. 547 were identified by substituting different carbon and nitrogen substrates for the yeast extract and casein in liquid screening medium, respectively. The results showed that *Planomicrobium* sp. 547 grew well with all of the carbon substrates tested, and could utilize ten of these to produce protease (Table 1). Regarding the utilization of nitrogen substrates, *Planomicrobium* sp. 547 grew well with all of the nitrogen substrates tested, but could only utilize casein to produce protease (Table 1).

Table 1 Carbon and nitrogen substrate utilization by *Planomicrobium* sp. 547

Carbon substrate	Activity /(μ/mL)	Nitrogen substrate	Activity /(μ/mL)
Arabinose	185	$(NH_4)_2SO_4$	0
Sucrose	154	NH_4Cl	0
Xylose	148	KNO_3	0
Lactose	134	NH_4NO_3	0
Glucose	163	Urea	0
Maltose	166	Tryptone	0
Cellobiose	159	Casein	210
Galactose	115		
Yeast Extract	110		
Fructose	108		
Rhamnose	0		
Starch	0		

2.3 Characterization of *Planomicrobium* sp. 547 protease.

The optimum temperature for *Planomicrobium* sp. 547 protease was 35℃, which was lower than that of mesophilic proteases (Figure 2). The enzyme showed approximately 50% of its residual activity at 20℃ and 60℃. After 60 min and 120 min of incubation at 50℃, the protease retained about 60% and 40% of its residual activity, respectively. The *Planomicrobium* sp. 547 protease showed a broad pH

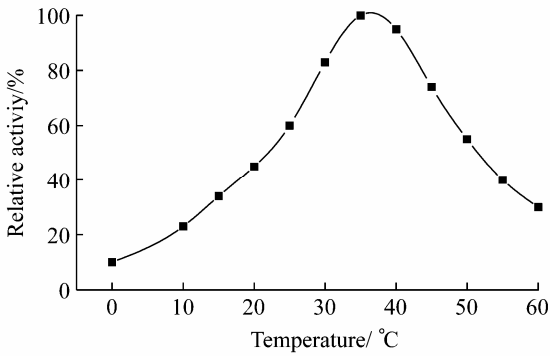

Figure 2 Effect of temperature on the *Planomicrobium* sp. 547 protease.

profile for casein hydrolysis with an optimal pH of 9.0 (Figure 3). The enzyme retained more than 80% of its activity between pH 7–11, and retained 50% of its residual activity at pH 12. These results indicated that the enzyme was an alkaline protease.

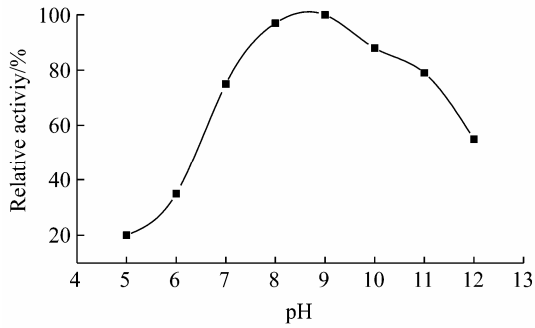

Figure 3 Effect of pH on the *Planomicrobium* sp. 547 protease.

Among the metal ions tested, Ca^{2+} and Mn^{2+} activated the enzyme, while Cd^{2+} and Co^{2+} obviously inhibited the enzyme. As shown in Table 2, The *Planomicrobium* sp. 547 protease activity was inhibited by 1 mmol/L phenylmethyl sulfonylfluoride (PMSF) and hydrochloride 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF), indicating that it was a serine protease. The enzymatic activity was strongly inhibited by low concentrations of SDS and guanidine HCl (1 mol/L), and high concentrations of urea (4 mol/L), indicating that hydrogen bonds may be important in maintenance of the enzyme activity. The enzyme was not sensitive to DTT or 2-mercaptoethanol, suggesting that disulfide bonds are not involved in preservation of the activity. The proteolytic activity was strongly inhibited by EDTA, indicating that the *Planomicrobium* sp. 547 protease was a metal protease.

Table 2 Effect of inhibitors and denaturing agents on protease activity

Reagent	Concentration	Relative activity /%
None	–	100
PMSF	1 mmol/L	7
AEBSF	1 m mol/L	2
SDS	1%	60
	2%	5
Urea	1 mol/L	90
	4 mol/L	65
	8 mol/L	15
HCl guanidine	1 mol/L	20
	4 mol/L	6
DTT	1 mmol/L	89
	10 mmol/L	6
2–Mercaptoethanol	0.1%	78
	5%	14
EDTA	1 mmol/L	60

2.4 Analysis of *Planomicrobium* sp. 547 protease gene

A 1 269 bp long open reading frame (ORF) encoding a serine protease was cloned from *Planomicrobium* sp. 547 using the PF and PR primers. The primary structure of the protease consisted of 421 AA residues. A conserved domain (309 AA) belonging to the peptidase S8 family domain was identified by searching the CDD (Conserved Domain Database). This domain contained a catalytic triad (Asp–145, His–185 and Ser–361) that is highly conserved in subtilase[11]. A search for homologous proteins in the GenBank showed that the *Planomicirobium* sp. 547 protease was 93% and 88% homologous with two subtilisins from *Bacillus* TA39 [12] and TA41[13], respectively. Interestingly, these three proteases all came from psychrophilic bacteria that were isolated from Antarctic samples. Furthermore, these three proteases, together with a subtilisin from psychrophilic *Bacillus* S41[14] formed an independent clade I (Figure 4). These results indicated that the evolution of this gene may be affected by the environment.

3 Discussion

In general, two bacteria can be identified as members of the same genus if they share more than 99% homology in their 16S rRNA gene sequence [15–16]. In this study, the 16S rRNA sequence of strain 547 showed more than 99% homology with that of both *Planococcus* and *Planomicrobium*. However, phylogenetic analysis revealed that strain 547 and other *Planomicrobium* strains were located in the same clade, which was separate from the *Planococcus* clade, indicating that strain 547 belonged to the *Planomicrobium* genus. Some *Planococcus* strains have been re–identified as *Planomicrobium*[17–19], indicating that these genera have a high degree of similarity. Based on the alignment and phylogenetic analysis, strain 547 was placed in genus *Planomicrobium* as *Planomicrobium* sp. 547.

The *Planomicrobium* sp. 547 protease exhibited characteristics typical of subtilase. The subtilases have been classified into six families based on their AA sequences: subtilisin, thermitase, proteinase K, lantibiotic peptidase, pyrolysin, and kexin[11]. Most of the highly conserved residues in subtilase were found in the *Planomicrobium* sp. 547 protease, including the catalytic triad residues Asp, His and Ser. Phylogenetic analysis demonstrated that the protease belongs to subtilisin. Other proteases in clade I (Figure 4) were all from *Bacillus*, implying conservation of the subtilisin sequence among different species of microorganisms. This is the first report of subtilisin isolated from the genus *Planomicrobium*. It should also be noted that the subtilisins in clade I (Figure 4) were all isolated from psychrophilic bacteria from Antarctic samples[12–13], except for *Bacillus* sp. S41[14]. The relationship between the genetic evolution and the environmental conditions of their habitat warrants further study.

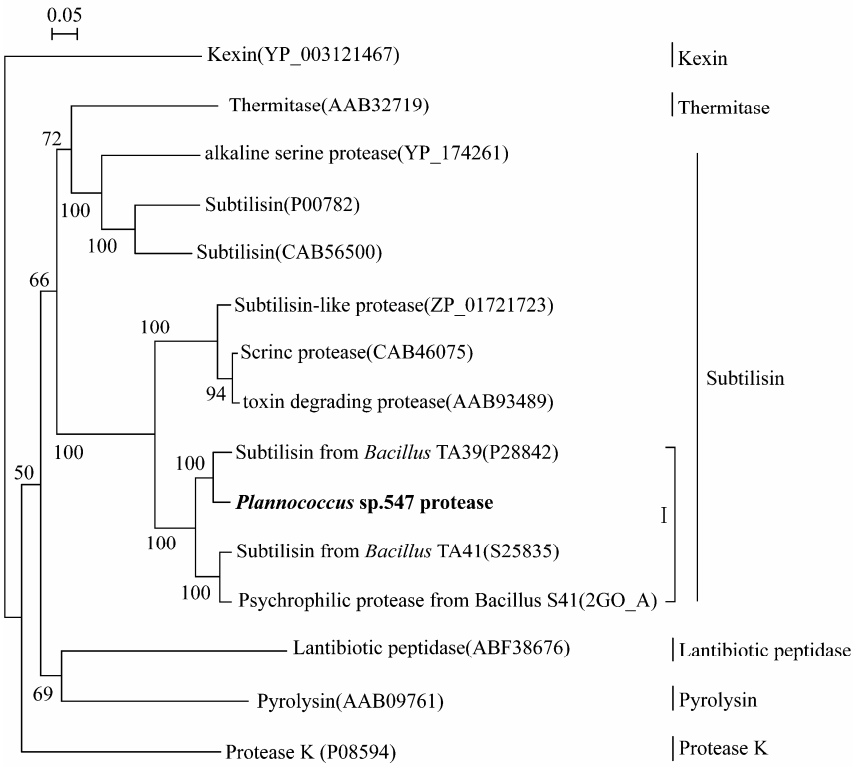

Figure 4 Phylogenetic analysis of *Planomicrobium* sp. 547 protease.

When compared to the subtilisin Carlsberg, which is another subtilase with wide application, the *Planomicrobium* sp. 547 protease showed several different characteristics. For example, the Carlsberg subtilisin also has an optimum pH of 9.0, and exhibited the highest levels of activity at 60℃; however, the *Planomicrobium* sp. 547 protease had a broader pH profile. Specifically, it retained more than 80% of its activity between pH 7 and 11 and 50% of its residual activity at pH 12. Moreover, the *Planomicrobium* sp. 547 protease showed high activity at temperatures below 35℃, and retained about 40% of its residual activity after 120 min of incubation at 50℃. All of these characteristics of *Planomicrobium* sp. 547 protease demonstrate its potential for industrial applications.

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