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Full Length Research Paper

Cloning and characterization of a psychrophilic catalase gene from an antarctic bacterium

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The gene of psychrophilic catalase BNC from Antarctic *Bacillus* sp. N2a was cloned by degenerate PCR and inverse PCR. BNC gene revealed a 1,461 bp open reading frame for a protein with 486 amino acids. The phylogenetic tree based on the amino acid sequences of BNC and representative psychrophilic microbial catalases manifested that BNC belonged to the Group III of the monofunctional catalase. The active-site residues of the structure-determined catalase were highly conserved in BNC. Comparison of the amino acid composition of BNC with its mesophilic homologue from *Bacillus subtilis* TE124 showed that BNC had properties of a cold-active enzyme.

Key words: Antarctic *Bacillus*, monofunctional catalase, cold-adapted enzymes, gene cloning.

INTRODUCTION

All organisms that survive in oxic environment are exposed to external or internal hydrogen peroxide which can inflict damage anywhere in a cell or tissue (Klotz and Loewen, 2003). Catalase (EC 1.11.1.6) can efficiently eliminate hydrogen peroxide to avoid oxidative harm to DNA, proteins or lipids. Catalase is an important protein in the cell metabolism of the aerobic microorganisms. Generally, catalases can be placed into three main groups, namely, monofunctional catalases, catalase-peroxidases and Mn-catalase. Monofunctional catalases are widely spread in three domains of life and are the most extensively studied (Klotz and Loewen, 2003).

Psychrophilic microorganisms are adapted for protection against hydrogen peroxide, which is important for survival at low temperatures where the solubility of oxygen is increased (Margesin et al., 2007). Marine psychrophilic bacteria *Colwellia psychrerythraea* achieves enhanced antioxidant capacity through the presence of catalase (Methe et al., 2005). Catalase genes are also in the complete genome of marine cold-adapted bacteria *Desulfotalea psychrophila* and *Pseudoalteromonas haloplanktis*. About 90% of the ocean volume is below 5℃ (Margesin et al., 2007), so studies on psychrophilic catalases are of significance to understand the metabolism of marine psychrophiles.

Antarctic ozone hole and the consequent increment of ultraviolet (UV) irradiance produce hydrogen peroxide in the aquatic environments. It can follow that aerobic bacteria in Antarctic surface water have high catalase activity in order to decompose hydrogen peroxide (Hernandez et al., 2002).

We isolated the strain *Bacillus* sp. N2a with high catalase activity and purified the catalase BNC (Wang et al., 2008a, b), which was the first characterized psychrophilic catalase from Gram-positive bacteria. In this paper, we describe the characterization of the BNC gene from the N2a strain aimed toward further understanding the psychrophilic catalase.

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Abbreviations: BNC, Catalase from *Bacillus* sp. N2a; **PCR,** polymerase chain reaction.

MATERIALS AND METHODS

The *Bacillus* sp. N2a strain was grown in marine broth (Wang et al., 2008a). Genomic N2a DNA was isolated as described before (Cheng and Jiang, 2006), and used as a template for PCR

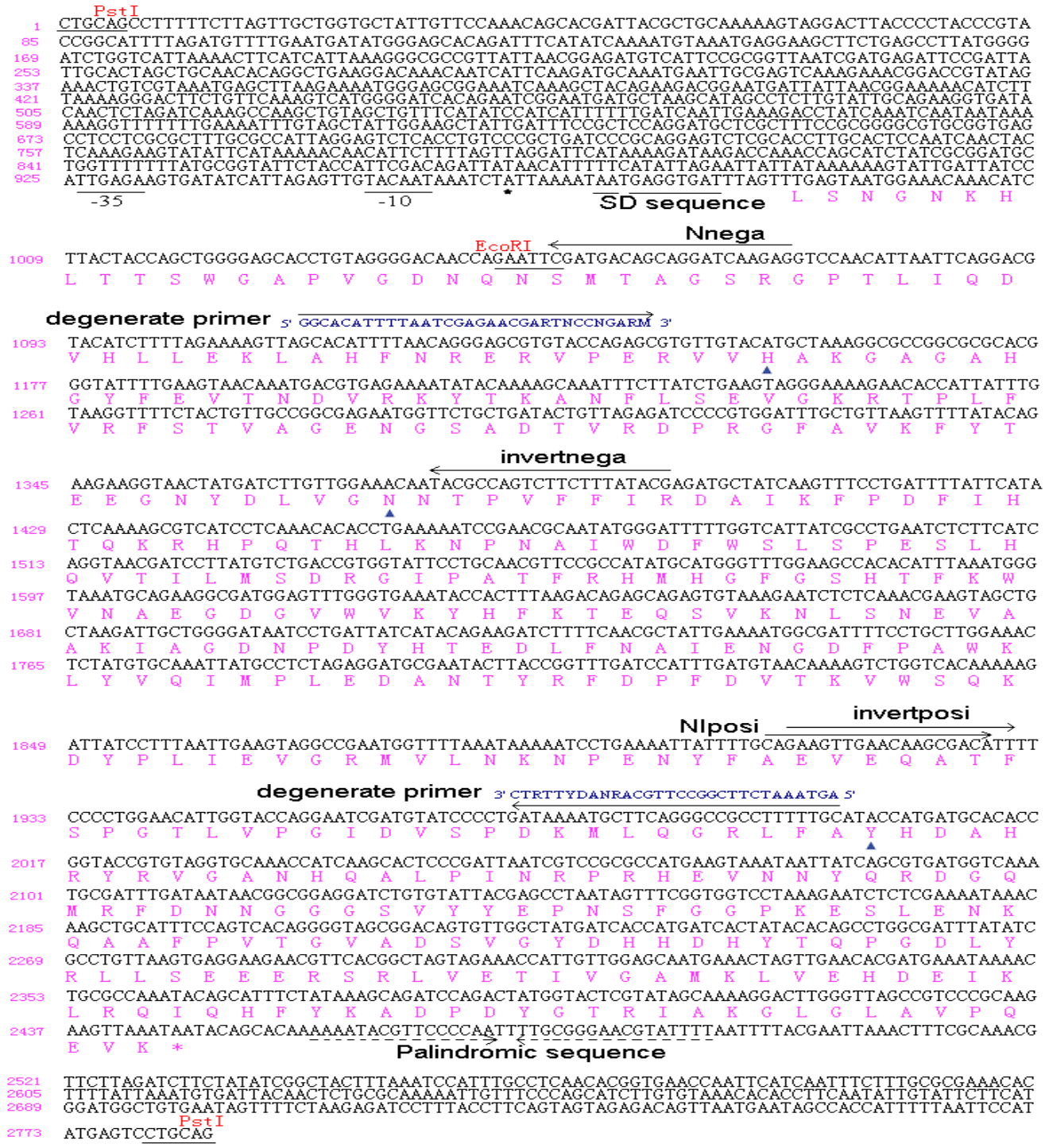

Figure 1. Nucleotide sequence of the *Bacillus* sp. N2a catalase structural gene and deduced amino acid sequence of the catalase. The putative promoter (the -35 and -10 regions) is underlined, as is the putative ribosome binding site (SD sequence); the transcription start site is marked by an asterisk. The putative intrinsic terminator (an inverted repeat capable of forming a stem-loop structure) is marked by dashed arrows. The conserved catalytic residues (HNY) are marked by triangles. The degenerate primer pair and two inverse primer pairs (invertposi/invertnega, NIposi/Nnega) were also showed.

amplification of a partial fragment encoding the catalase. Degenerate oligonucleotides primers (Figure 1) were designed by CODEHOP (http://blocks.fhcrc.org/codehop.html) based on the amino acid sequences of the conserved regions, which were obtained from the multiple sequence alignment from known catalases. The PCR was carried out using 30 cycles of 94℃ for 30 s, 55℃ for 60 s and 72℃ for 60 s, and an extension of 72℃ for 5 min.

Inverse PCR was carried out as described before (Sambrook and Russell, 2006) with some modifications. We used a 25 µL reaction volume and the ligated DNA was directly used as the PCR template without extraction. The primers (Figure 1) were designed by Primer Premier 5 (Palo Alto, US) according to the known BNC gene fragment. The N2a genome DNA was digested by EcoRI and the inverse PCR was carried out with the primer pair invertposi and invertnega using 30 cycles of 94℃ for 30 s, 45℃ for 30 s and 72℃ for 2.5 min, and an extension of 72ºC for 10 min. Then the N2a DNA was digested by PstI and PCR was carried out with the primer pair Nnega and NIposi using 30 cycles of 94ºC for 30 s, 45ºC for 1 min and 72℃ for 3 min, and an extension of 72℃ for 10 min.

The resulting PCR product was subcloned into pGM-T (Tiangen, Beijing, China). The sequence was determined by automated DNA sequencing in Sangon (Shanghai, China). Restriction enzymes, ligase and Taq enzymes were purchased from Fermentas (Shenzhen, China).

The ribosomal binding site of BNC gene was predicted according to the 3' end sequence of *Bacillus subtilis* 16S rRNA (Osada et al., 1999). The promoter sequence was predicted by BDGP (http://www.fruitfly.org/seq_tools/promoter.html). Catalase sequence alignments were made using PROMALS3D (http://prodata.swmed.edu/promals3d/promals3d.php). The amino acid composition of the catalases were calculated and phylogenetic evolutionary analyses were conducted using MEGA version 5 (http://www.megasoftware.net/).

The BNC gene was amplified by PCR with the primers corresponding to the N-terminus and the C-terminus with NcoI and XhoI restriction site sequences, respectively. The PCR product, digested with NcoI and XhoI, was ligated into pET28 and the BNC gene in the resulting plasmid was confirmed to have the correct sequence. The plasmid was transformed into *Escherichia coli* BL21 (DE3) cells and the expression method was according to Novagen pET system manual 11th edition. The catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm resulting from the elimination of hydrogen peroxide (Wang et al., 2008b). The sequences of the BNC gene and the deduced protein have been filed in the GenBank (Accession No. FJ665431 and ACN 54670).

RESULTS AND DISCUSSION

Among all the degenerate primer pairs, only the one producing the longest gene fragment (PCR product 827 bp) was shown in Figure 1. Besides EcoRI and PstI, other restriction endonucleases such as BamHI, SalI, XhoI and HindIII were also used but failed. The inverse primers invertposi and invertnega were used for amplifying the unknown catalase gene fragment. But the primer invertposi failed to amplify the BNC gene and was superseded by NIposi. According to the new 99 bp sequence obtained by the primer invertnega, Nnega primer was designed. Nnega and NIposi successfully amplified the N/C end and the upstream (986 bp)/downstream (338 bp) sequences of BNC. The catalase coding sequence (CDS) encoded a protein of 486 amino acids initiated with a TTG codon at nucleotide 987 and terminated with a stop (TAA) codon at nucleotide 2445. The monomer molecular mass was predicted to 55,075 Da which was similar to that reported earlier (Wang et al., 2008b). A potential Shine–Dalgarno (SD) sequence or ribosome-binding site was identified at positions -16 to -6 upstream of the TTG initiation codon. The putative -35 and -10 promoter areas were identified at positions -45 to -40 and -20 to -15, respectively, upstream of the potential SD sequence.

Percentage utilization of TTG initiation codon in 16 *Bacillus* chromosomes was from 7.8% to 16% (Villegas and Kropinski, 2008). So TTG codon-initiated genes were common in *Bacillus* genomes. Besides BNC, the catalase gene KatX1 (CP000813) in *Bacillus pumilus* SAFR-032 and KatX (NC_000964 REGION: 3964997.3966640) in *B. subtilis* subsp. *subtilis* str. 168 were both initiated by TTG.

Overexpression of recombinant BNC in *E. coli* BL21 (DE3) showed distinct catalase activity in the supernatant of the sonication-disrupted cells, which was 10 times the activity of the controlled *E. coli* BL21 (DE3) with pET28. The recombinant BNC catalase activity confirmed the function of the cloned gene.

Protein sequence analysis by BLAST (http://blast.ncbi.nlm.nih.gov) revealed that the BNC displayed the highest percentage identity (90%) to catalase KatE (ADF42025) from *Bacillus megaterium* DSM319 genome. Among the purified and characterized catalases, BNC had the highest identity (89%) to the catalase (BAB21251) from *B. subtilis* TE124 (Ni et al., 2001). BNC was also aligned with the catalases in protein data bank (PDB, http://www.rcsb.org/pdb) in which BNC had the highest identity (68%) to the catalase (PDB ID 1SI8) of *Enterococcus faecalis*.

Monofunctional catalases could be divided into three groups, i.e, Group I, Group II and Group III based on the phylogenetic relationship (Klotz and Loewen, 2003). The phylogenetic tree of BNC and other psychrophilic catalases (Figure 2) demonstrated that BNC belonged to the Group III, of the monofunctional catalases and the cold-adapted catalases existed in all three groups of the monofunctional catalase. The psychrophilic catalase sequences in the phylogenetic tree were from the whole genome sequences of psychrophilic prokaryotes except BNC and two catalases from *Vibrio* and *Aliivibrio*, of which their genes had been cloned (Ichise et al., 2000; Lorentzen et al., 2006). Group III, was the largest group of the three groups (Klotz and Loewen, 2003) and also contained most reported psychrophilic catalases which is mainly the catalases from Proteobacteria, while BNC and its mesophilic homologues from *B. subtilis* TE124 and *E. faecalis* was from Firmicutes, a phylum of Gram-positive bacteria, which constituted a minor subgroup in Group III. Chances were that more complete genome sequences of psychrophilic Gram-negative Proteobacteria had been determined than those of Gram-positive bacteria.

The highly conserved catalytic residues (H56, N128 and Y339) of BNC were the same as other mono-functional catalases (Zamocky et al., 2008), suggesting that BNC have the same or similar catalytic mechanism as/to the catalases with experimentally-determined structures. Comparison of the amino acid composition of BNC with its mesophilic homologue from *B. subtilis* TE124 showed that BNC had properties of a cold-active enzyme (Siddiqui and Cavicchioli, 2006), such as a lower

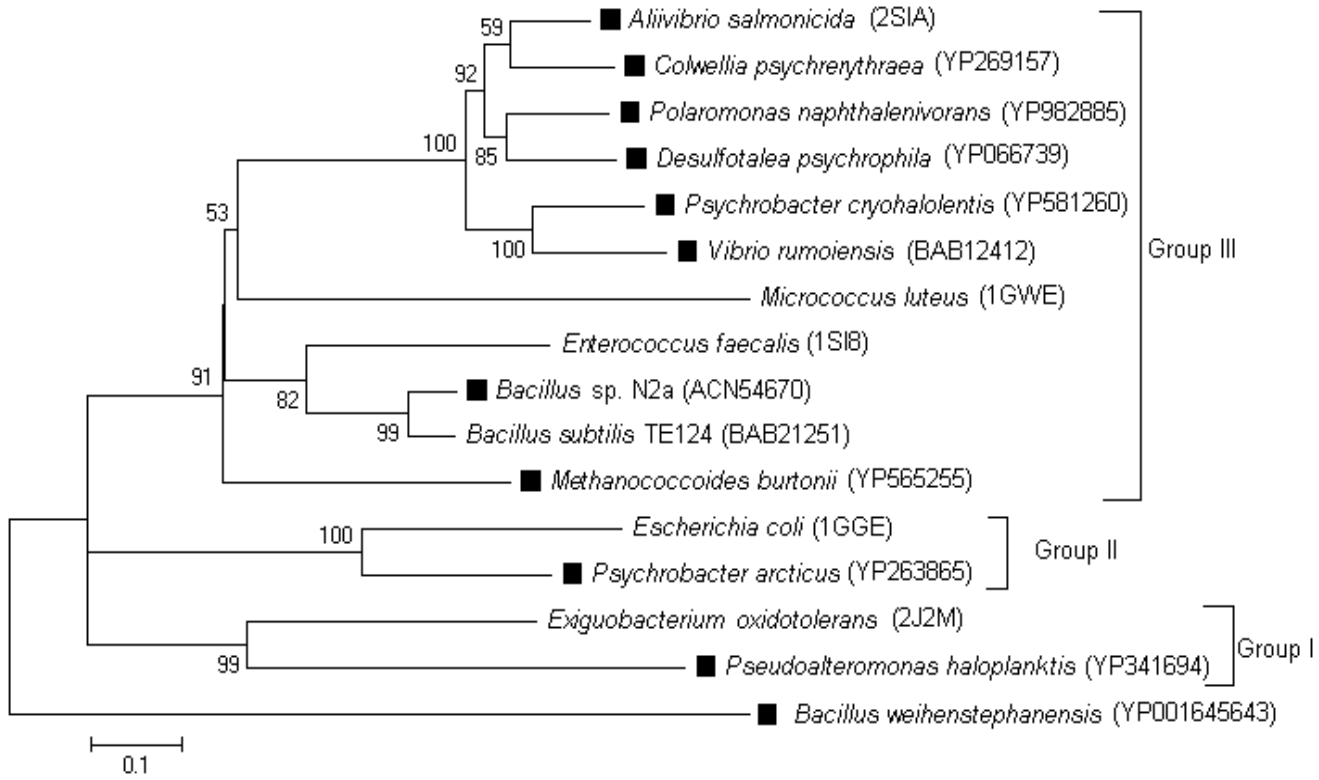

Figure 2. Phylogenetic tree based on the amino acid sequences of 16 monofunctional catalases illustrating the relationship of BNC to other related catalases. Psychrophilic catalases were marked by squares. The tree was inferred by using the maximum likelihood method based on the Whelan and Goldman model (WAG). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank and PDB accession numbers were indicated.

isoleucine content (3.5% versus 4.1%), less net charged residues (115 versus 124), a higher glycine content (8% versus 7.7%) and a higher histidine content (4.7% versus 3.7%). However, the amino acid sequence of BNC could directly not reveal cold-adapted features on the molecular level. Our ongoing crystallographic studies of BNC and eventual establishment of its molecular structure will provide further information about the catalytic function of psychrophilic catalases.

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