A Method for Molecular Analysis of Catalase Gene Diversity in Seawater

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Abstract Catalase plays an important role in the metabolism of marine bacteria and has potential impact on the marine environment. Four PCR primers were designed to amplify the catalase gene fragments in marine bacteria by applying metagenomic DNA from Yellow Sea surface water as the template. Of the four reproducible target PCR products, the longest one with 900 bp were chosen for catalase gene library construction by the T-vector and the white *Escherichia coli* colonies in the library was screened through restriction-digesting the reamplified insert fragments by the selected restriction endonuclease *Mbo*I, and then the bands of the resulting products were displayed in the agarose gel by electrophoresis. The unique restriction fragment length polymorphism (RFLP) pattern was selected and the corresponding catalase gene fragments were sequenced, which verified that every unique RFLP pattern represented one type of catalase. This PCR–RFLP method above was established to investigate the bacterial catalase diversity in seawater.

Keywords Marine bacteria · Hydrogen peroxidase · PCR–RFLP method · Surface seawater

Introduction

Hydrogen peroxide emerges in the life process especially the aerobic metabolism. As one of the reactive oxygen species (ROS), hydrogen peroxide is a strong oxidizing agent and could harm almost all cell components. Catalase (EC1.11.1.6) is a kind of housekeeping enzyme in the microorganisms in the oxic environment, which decomposes hydrogen peroxide rapidly and efficiently in order to protect biological molecules from oxidative damage. Catalases can be phylogenetically divided into three groups, namely, monofunctional catalases, catalase-peroxidases and Mn-catalase. Monofunctional catalases are widely spread in prokaryotes and eukaryotes and represent the largest group of hydrogen peroxide-degrading enzymes, which falls into three member clades including small-subunit enzymes (clade 1 and clade 3) and large-subunit enzymes (clade 2) [1].

Ninety percent of the ocean volume is below 5 °C where the solubility of oxygen is increased. Consequently, marine aerobic microorganisms are adapted for protection against ROS with their imperative antioxidant catalases [2]. Catalase genes are in the complete genomes of marine cold-adapted bacteria *Desulfotalea psychrophila*, *Colwellia psychrerythraea* and *Pseudoalteromonas haloplanktis* et al. so catalases are of significance to understand the metabolism of marine psychrophiles.

Hydrogen peroxide concentrations in surface seawater are influenced by ultraviolet and atmospheric wet deposition, which might reach micromolar concentrations [3]. Marine bacteria in the surface waters exposed to external H₂O₂ face an oxidative stress situation. It can follow that they have enough catalase activity in order to decompose H₂O₂ [4]. Moreno held that the catalase from marine microorganism in the surface seawater broke down H₂O₂

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produced by UV radiation, which was a biogeochemical process releasing heat and accelerating sea ice melting with important effects on the ecosystem [5]. Catalases also have potential to function as biomarkers for stress-induced enhanced robustness in psychrotolerant bacteria [6]. The molecular evolution of catalase showed evidence for horizontal gene transfer [7], which might further the understanding of the genome evolution of the marine microorganisms [8].

It is necessary to establish a catalase diversity-detecting method for further understanding the ecological role of the catalase in marine microorganisms. The activity of the catalase in seawater could be measured directly [9] but this is not an effective method to study the amount or variety of the marine microbial catalase. The catalase diversity could be studied by metagenomic construction [10], while the cost of metagenomic library construction and sequencing are fairly high. Although the specific and conserved PCR primers for amplifying the target genes are widely applied to study gene diversity in environmental samples, yet the PCR primers for amplifying catalase genes in seawater have not been reported to our knowledge. The polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) technique has been adopted for bacterial diversity study [11, 12]. We designed degenerate catalase primers and employed molecular techniques to address the catalase diversity of the Yellow Sea surface water through PCR–RFLP and sequencing.

Materials and Methods

Design of Degenerate PCR Primers

Degenerate oligonucleotides primers were designed by CODEHOP (<http://blocks.fhcrc.org/codehop.html>) [13] based on the amino acid sequences of the conserved regions obtained from the multiple sequence alignments from known bacterial catalases in GenBank. The multiple sequence alignments were produced by PROMALS3D software [14].

Sample Collection, DNA Extraction and PCR

The Yellow Sea surface seawater samples (36°00'N, 124°30'E) were collected in January of 2011 and stored for 3 weeks in dark at 4 °C before use. Seawater (4 L) was prefiltered through 8- μ m pore-size glass fiber filters and then filtered onto 50-mm diameter 0.22- μ m polysulfone filter at a pressure <0.03 MPa. The seawater DNA was extracted as previously described [15].

PCR amplification of bacterial catalase gene sequences was performed with the Tprofessional standard gradient 96

thermocycler (Biometra, Germany). Final concentration of PCR reagents are as follows, 1 $\times$ EasyTaq DNA polymerase buffer (TransGen Biotech, China), 0.04 % bovine serum albumin, 200 μ M dNTP mix, 600 μ M each forward and reverse primers, 1.2 U Taq DNA polymerase, and 2 μ l metagenomic DNA; PCR grade water was added to bring the reaction volume to 25 μ l. Thermocycling parameters were a modification of conventional touchdown PCR method. Initial denaturation was for 2 min at 94 °C: stage 1, 94 °C 30 s, 60–51 °C 60 s (–1 °C per cycle), 72 °C 90 s for 10 cycles; stage 2, 94 °C 30 s, 50 °C 60 s, 72 °C 60 s for 20 cycles. A final primer extension of 3 min was added after stage 2. PCR products from six reactions were pooled to minimize PCR bias, gel-purified by Sanprep Kit (Sangon, China) and ligated into pBS-T vectors (Tiangen, China) to transform *Escherichia coli* TOP10 competent cells. Recombinants were selected using X-Gal-IPTG LB indicator plates with 100 μ g/mL ampicillin.

RFLP and DNA Sequencing

All the white macroscopic clones were chosen and the positive ones were verified by the catalase gene fragments reamplified from these clones using the degenerate primers, then those PCR products were digested by the restriction endonuclease selected as described somewhere [16] with some modifications.

Enzymes which generated DNA bands of similar size from various bacteria were rejected as this might hide the complexity of the catalase gene pool, and in the resulting products, the fragments smaller than 50 bp were neglected for they were hard to detect. Theoretical digestions were simulated from 15 sequences of the monofunctional catalase genes of three types in the GenBank: *Pseudomonas syringae*, accession number NC 007005; *Exiguobacterium oxidotolerans*, AB 266484; *Escherichia coli*, M55161; *Sinorhizobium meliloti*, NC 003078; *Aeromonas veronii*, EF028076; *Leptospira interrogans*, AE011360; *Vibrio salmonicida*, DQ182487; *Bacillus subtilis*, M80796; *Bacteroides fragilis*, U18676; *Bordetella pertussis*, U07800; *Brucella abortus*, U11439; *Campylobacter jejuni*, X85130; *Haemophilus influenzae*, U02682; *Helicobacter pylori*, U67458; *Bacillus* sp. N2a, FJ665431. The first two sequences belonged to clade 1 and the next two sequences were in clade 2 and the other 11 sequences were from clade 3.

The above bacterial monofunctional catalase gene fragments between the positive and negative degenerate primers were selected out [17], simulatedly digested and electrophoresed by the online NEBcutter software (<http://tools.neb.com/NEBcutter2>) [18]. And then the restriction enzymes recognizing DNA sequences of 4 bp that theoretically should distinguish different species were identified.

Table 1 Degenerate primers for amplifying small-subunit monofunctional catalases

	Primer name	Nucleotide sequences of degenerate primer	Conserved amino acids corresponding to primer*	Reference
Positive	C3 ⁺	GGCACATTTTAATCGAGAACGARTNCCNGARMG	AHFNRRERIPER(41–51)	[13, 21]
	cat1	CCNGARMGNGTNGTNCAYGC	PERVVHA(49–55)	[22]
Negative	C3 [−]	AATCTTTATGAGGCCAAACTTTGTNANRTCRAA	FDLTKVWPHKDY(276–287)	[13]
	CB [−]	AGTAAATGTTCCGGCCTTGCARNADYTTRTC	DKMLQGRIFS(327–336)	[13, 21]

* The numbers indicates the location of the conserved amino acids in the *Bacillus subtilis* catalase (GenBank accession number NP 388762)

Fig. 1 Catalase gene fragments from Yellow Sea surface water metagenomic DNA were amplified by four degenerate primers. M DNA marker, 1 cat1C3[−], 2 cat1CB[−], 3 C3⁺CB[−], 4 C3⁺C3[−]

PCR products of positive clones were restriction-digested with the selected enzyme (Fermentas, Thermo Fisher Scientific Inc.), and restriction fragments were resolved by electrophoresis on 1 % agarose gels in 10 mM Li⁺–H₃BO₃, pH 6.5 [19]. Band patterns digitally photographed with an imaging system (Peiqing Biotech, China) were compared for RFLP analysis to identify redundant clones. Every clone of typical RFLP type was chosen and the gene fragment was sequenced (Sangon, China). Catalase sequences were analyzed by BLAST (<http://blast.ncbi.nlm.nih.gov>) and the phylogenetic groups of the enzymes were determined by MEGA 5 [20].

Results and Discussion

It was difficult to design degenerate primers according to the conserved regions of catalase-peroxidases, Mn-catalase and clade 2 of monofunctional catalases. Four degenerate primers (Table 1) for clade 1 and 3 of monofunctional catalases, however, could be designed and the four primer pairs were capable of reproducibly amplifying metagenomic catalase gene fragments (Fig. 1). While not brighter than other three bands on the agarose gel, the resulting DNA band produced by C3⁺CB[−] primers was of the largest size (~900 bp) in accordance with the expectation. So this PCR product was chosen for library construction and RFLP analysis of catalase diversity.

Fifteen monofunctional catalase gene fragments between the primers C3⁺ and CB[−] were digested and electrophoresed online by restriction endonuclease recognizing 4 bp and *Mbo*I was chosen as the best enzyme for differentiating those catalases derived from different bacteria.

The metagenomic catalase gene library constructed by the PCR product of C3⁺CB[−] primers was screened and a total of 25 out of the 40 clones from the gene library showed the correct fragment size. Then the positive PCR products were singled out for further RFLP analysis by the *Mbo*I enzyme. The resulting restriction-digested PCR products displayed seven unique electrophoresis band patterns and these RFLP types were confirmed to be corresponding to seven operational taxonomic units after sequencing and translation of all the 25 inserts.

Twenty-five gene fragments of catalases in Yellow Sea surface water were sequenced including seven types of catalases (Table 2; Fig. 2), five types of which were derived from Proteobacteria (20 sequences) and two types of which belonged to Firmicute catalases (5 sequences). The degenerate primers guaranteed that all the amplified genes belonged to small-subunit catalases, but only one type of catalase belonged to clade 1 and other six types of enzymes belonged to clade 3.

The results above suggested that the clade 3 monofunctional catalase of Proteobacteria might be predominant in the Yellow Sea surface water, which was in accordance with the widespread distribution of Proteobacteria in the ocean [23] and high prevalence of clade 3 monofunctional catalase in nature [1]. The number of small-subunit monofunctional catalase was more than the sum of catalase-peroxidases, Mn-catalase and large-subunit monofunctional catalase [7], and therefore diversity and abundance of catalase genes in the sea could be inferred by detecting the representatives, i.e. small-subunit monofunctional catalase genes.

Conclusions

All of the four degenerate primers in this study could be used for amplifying small sub-unit monofunctional catalase genes from seawater metagenomic DNA. The C3⁺CB[−]

Table 2 Catalase gene fragments from Yellow Sea surface seawater obtained by PCR with C3⁺CB[−] primers

Catalase name	Most similar catalase in Genbank by BLASTx search	Classification of catalase-owning strain	Group of monofunctional catalase	Number of catalases
YS9.30.6	<i>Nitrococcus mobilis</i> Nb-231 catalase ZP_01126602	Gamma proteobacteria	Clade 3	7
YS9.30.3	<i>Marinomonas</i> sp. MED121 catalase ZP_01077644	Gamma proteobacteria	Clade 3	6
YS10.15.4	<i>Alteromonas macleodii</i> ATCC 27126 catalase ZP_04715255	Gamma proteobacteria	Clade 3	4
YS10.6.2	<i>Ferrimonas balearica</i> DSM 9799 catalase YP_003911930	Gamma proteobacteria	Clade 3	2
YS9.9.8	<i>Nitrosomonas europaea</i> ATCC 19718 catalase NP_841908	Beta proteobacteria	Clade 3	1
YS9.30.7	<i>Oceanobacillus iheyensis</i> HTE831 catalase NP_691554	Firmicutes	Clade 3	3
YS9.30.10	<i>Bacillus licheniformis</i> ATCC 14580 catalase YP_081189	Firmicutes	Clade 1	2

Fig. 2 Catalase gene restriction patterns from Yellow Sea surface seawater displayed as a result of in silico restriction enzyme *Mbo*I and 2 % agarose gel electrophoresis. The catalase names were in accordance with those in Table 2. The PCR products in Table 2 were simulatedly digested and electrophoresed by the online NEB-cutter software [18]

primer pairs, however, could produce the PCR results with the largest gene fragment. Although the nonspecific PCR amplification sometimes occurred (data not shown), the false positive clones could be ruled out by electrophoresis and sequencing. The above results of sequencing all the positive insert catalase gene fragments showed that it was not necessary to sequence every gene every time for the restriction endonuclease *Mbo*I could effectively differentiate the unique C3⁺CB[−] fragment of the catalase genes (Fig. 2). And the amount and types of small-subunit monofunctional catalases could be detected by the PCR–RFLP method, which would be applied for studying the diversity of catalases in the seawater.

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