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Polymorphism of the superoxide dismutase gene family in the bay scallop (*Argopecten irradians*) and its association with resistance/susceptibility to *Vibrio anguillarum*

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ABSTRACT

The superoxide dismutases (SODs) are a family of enzymes that function as the first line of antioxidant defense against highly reactive superoxide radicals. The bay scallop *Argopecten irradians* contains three unique superoxide dismutases: Ai-icCuZnSOD, Ai-MnSOD and Ai-ecCuZnSOD, which were characterized in our previous studies. qRT-PCR was also performed to characterize the temporal expression of SODs in the hemocytes of bay scallops injected with the bacterium *Vibrio anguillarum*. To characterize the SOD family in *A. irradians* completely, we evaluated the polymorphism in the SOD genes and investigated the association of this polymorphism with resistance/susceptibility to *V. anguillarum*. Fifty-nine SNPs were identified in the promoter, exon and partial intron sequences of the three SOD genes. AiECSOD contained the most SNPs, as compared to AiCuZnSOD and AiMnSOD, and the majority of these were located in the promoter. Among them, the genotypes of −1739 T–C SNP in the AiCuZnSOD promoter and alleles of the −498 A–T and −267 G–A SNPs in the AiECSOD promoter showed a significant association with resistance/susceptibility to *V. angullarum* ($P < 0.05$). The only non-synonymous SNP that was identified, E1-38 C–A in Ai-ecCuZnSOD, was a dimorphism caused by a C to A transition that resulted in a Thr to Lys substitution at position 13 in the signal peptide. The Thr allele was associated with increased susceptibility to *V. anguillarum* ($P < 0.05$). To confirm the presumption, another independent challenge experiment was performed, in which the cumulative mortality of Ai-icCuZnSOD Q−−1739 genotype TT was significantly lower than TC ($P < 0.05$). Ai-ecCuZnSOD Q−−498 genotype AA and AT were significantly lower than TT ($P < 0.05$), Ai-ecCuZnSOD E1-3 genotype AA was significantly higher than CA and CC ($P < 0.05$). The results suggested that these three polymorphic loci could be potential gene markers for the future molecular selection of strains that are resistant to diseases caused by *V. anguillarum*.

1. Introduction

Marine bivalves are widespread in coastal environments and, due to their filter-feeding habits, they can accumulate large numbers of bacteria [1]. Bacterial diseases in molluscs have been described mainly in the larval stage and in adult molluscs, and there is a marked predominance of diseases caused by the genus *Vibrio* [2,3]. *V. anguillarum* is a ubiquitous marine bacterium in coastal waters [4], and it has long been recognized as pathogenic for multiple mollusc species, which include *Mytilus galloprovincialis* [5], *Argopecten prupuratus* [6], *Ruditapes philippinarum* [7], and *Crassostrea gigas* [8]. Therefore, it may be possible to utilize infections caused by the genus *Vibrio* to develop an experimental disease model in molluscs. It has been proved that when molluscs are attacked by microorganisms or viruses, one important immune defense reaction of molluscan hemocytes is phagocytosis [9]. During the course of phagocytosis, the host glycolytic pathway becomes activated, which in turn increases the consumption of oxygen and induces the production of reactive oxygen species (ROS) [10,11]. ROS can cause damage to DNA, including breakage, as well as lipid peroxidation, protein modification, membrane disruption, and mitochondrial damage [12,13].

The superoxide dismutases (SODs; EC 1.15.1.1) are the first and most important line of defense against ROS, and in particular

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superoxide anion radicals [14]. SODs are classified into four distinct groups according to their metal content. Three isozymes of *A. irradians* SOD (Ai-icCuZnSOD, Ai-MnSOD and Ai-ecCuZnSOD) were identified and reported in our previous studies [15–17]. Analysis by quantitative real-time reverse transcription-PCR (qRT-PCR) indicated that the level of SOD transcripts in the hemocytes of bay scallops increases significantly after the injection of *V. anguillarum*, which suggests that *V. anguillarum* triggers an immune response that involves Ai-SODs [15,16].

As the number of immune-related genes that have been cloned and characterized increases, there is a growing realization that our knowledge of gene polymorphism and disease susceptibility in mollusca is often inadequate. Thus, there is an urgent need to increase basic research into genetic polymorphisms and immunity phenotypes in a diversity of species. Several studies have suggested that an association exists between polymorphisms of SOD genes and human diseases. In MnSOD, the Val allele and Val/Val genotype is associated with an increased risk of lung [18] and bladder cancer [19]; the Ala allele is associated with an increased risk of breast [20,21], prostate [22,23], and ovarian cancer [24]. In ecCuZnSOD, the substitution of arginine at position 213 by glycine causes an 8–15-fold increase in the plasma levels of ecCuZnSOD [25,26]. These studies demonstrated that SODs play an important role in maintaining the health and metabolic stability of organisms. Although several SOD genes from aquatic animals have been cloned and characterized, there have been no reports about the association between SOD sequence polymorphisms and immunity phenotypes as yet. Our objective was to identify polymorphisms in three SOD isoforms of the bay scallop and to investigate the possible association of these polymorphisms with resistance/susceptibility to infection by the bacterium *V. angullarum*.

2. Materials and methods

2.1. Animals and bacterial challenge

Two hundred bay scallops that averaged approximately 60 mm in shell length were collected randomly from several scallop farms in Qingdao (Shandong province, China). Scallops were acclimatized in seawater tanks (10 m^3) for 1 week before processing. The seawater temperature was 18 ± 1.0 °C and the salinity was 30‰ throughout the experiments.

For the bacterial challenge experiment, the scallops were divided into five groups (40 scallops in each group). Four of the groups were cultured in 25 l of seawater that contained a high density of *V. anguillarum* (8×10^7 cfu/ml, strain no. MVM425). The remaining group was cultivated in seawater that did not contain bacteria and was used as the control group. All the scallops were monitored at hourly intervals during the first 12 h and then every 3 h until 7 days post-challenge to identify those that had died. The scallops that died during the early period of the challenge experiment were classified as susceptible stock and the individuals that survived throughout the challenge were classified as resistant stock. The adductor muscle of each scallop that belonged to these two stocks was removed and kept at −80 °C until the DNA was isolated.

2.2. Sampling and DNA isolation

To detect single nucleotide polymorphisms (SNPs) in the SOD genes and to determine whether the alleles were associated with resistance/susceptibility to *V. anguillarum*, adductor muscles were collected from the first 30 scallops to die (susceptible stock) and from the last 30 survivors of the bacterial challenge (resistant stock). Genomic DNA was purified from the adductor muscles using a Genomic DNA Purification Kit (Promega).

2.3. Primers and PCR amplification

Genomic DNA from the 30 susceptible and 30 resistant bay scallops was amplified via PCR. Gene-specific primers were designed based on the sequences of three SOD genes (Ai-icCuZnSOD, GenBank accession no. FJ014707; Ai-MnSOD, GenBank accession no. FJ590509; Ai-ecCuZnSOD, GenBank accession no. EU914002). The PCR amplification products ranged in size from 300 bp to 700 bp. The amplifications were performed in a total volume of 50 μl, which contained 5 μl of 10× PCR buffer, 3 μl of $MgCl_2$ (25 mM), 1 μl of dNTP mix (10 mM), 2 μl of each primer (10 μM), 37.6 μl of PCR grade water, 0.4 μl (1 U) of Ex-Taq polymerase (TaKaRa, Japan) and 100 ng of DNA template. The PCR was carried out with the following conditions: 35 cycles of 94 °C for 40 s, *n* °C (depended on the annealing temperatures of the different primers) for 40 s and 72 °C for 50 s, and a final extension of 72 °C for 10 min. After amplification, the PCR products were detected by electrophoresis on 1.5% agarose gels. Primers that amplified single specific bands were chosen for SNP analysis (Table 1). Specific PCR products were purified and sequenced. The promoter, exons and portions of the introns at the exon/intron boundaries of the bay scallop SOD genes were amplified and sequenced. All DNA variations, which were only observed in only one individual, were confirmed by new PCR followed by resequencing.

2.4. Identification and analysis of SNPs in bay scallop SOD genes

The nucleotide sequences of the SOD genes from different individual scallops were aligned using DNAMAN and polymorphisms in the genes were detected from these alignments. Given that polymorphisms were identified in the promoter regions of the SOD genes, TRANSFAC and AliBaba 2.1 software (http://www.gene-regulation.com/pub/programs/alibaba2/index.html) was used to predict putative binding sites for transcription factors within these regions. Statistical analysis was carried out with SPSS 13.0. The χ^2

Table 1
Primers used for SNP analysis in three SODs.

Primer	Sequence (5′–3′)	Application	Product
CuZnSOD-SNP-F1	AATCACCTTTCAATACGCAAACAGC	Promoter	775 bp
CuZnSOD-SNP-R1	CTTGTGGTACCTGGCAATTCAAAGA		
CuZnSOD-SNP-F2	AGACAATAAAGTTCATTTGACAGGA	Exon2 and	375 bp
CuZnSOD-SNP-R2	TAACCTAAAGTGACTGAGTATGCTA	intron3	
CuZnSOD-SNP-F3	ATGGTGGTAAGTTGGTTATCCCTAT	Intron3 and	549 bp
CuZnSOD-SNP-R3	CAAACATACAGCACATACTGGTCAA	exon4	
MnSOD-SNP-F1	TAGGAGCTTATGAAGTACGCAAATC	Promoter	452 bp
MnSOD-SNP-R1	CCCTTGTGTCGATGTACAGACTTTA		
MnSOD-SNP-F2	CGCCTTATTACTTCCTTGAAACACT	Promoter	564 bp
MnSOD-SNP-R2	AAAAGAAACGAACAGAACAAAAATA		
MnSOD-SNP-F3	TTTTTGTTCTGTTCGTTTCTTTTCT	Exon1 and	512 bp
MnSOD-SNP-R3	ACAGTCAGCAAACTAATGTCCGATA	intron1	
MnSOD-SNP-F4	TTAGATGGACACAAAAAGAAAAGGA	Intron2	345 bp
MnSOD-SNP-R4	AAATGAAAGGTTGGTAGGTAGGGAT		
MnSOD-SNP-F5	GAGTTCCACCTTTTGATATAGCCCT	Exon2	385 bp
MnSOD-SNP-R5	GCATTGTAGTCATAGGGAAGGTCTG		
MnSOD-SNP-F6	ACACGATTTATCACTGATCCATGCT	Exon3	463 bp
MnSOD-SNP-R6	ACAACCCATATCCAGACCTACACAA		
MnSOD-SNP-F7	TTGGCTACCACATACTTGATTTTAG	Exon4	715 bp
MnSOD-SNP-R7	TTATACAGTGTTGTAGCGATCAAGC		
ECSOD-SNP-F1	GAAAACGCGCGAGAGATCTTAGAGT	Promoter	560 bp
ECSOD-SNP-R1	TTTCTGTGGATTCACTGTTGTCTTC		
ECSOD-SNP-F2	TCTCAAGAAGACAACAGTGAATCCA	Promoter	379 bp
ECSOD-SNP-R2	ACTATTCGCTGTGATTTTTTTCTGC	and exon1	
ECSOD-SNP-F3	TCTACCAGAATATCGGTGAGATGAA	Exon2	453 bp
ECSOD-SNP-R3	TGTTGAATGGCCTTCTACCTTCTAT		
ECSOD-SNP-F4	ACTTTCGGGAATCCATCTTTACC	Exon3	419 bp
ECSOD-SNP-R4	ACCTACATTTTGAAGAGTCGCAC		
ECSOD-SNP-F5	CGATAGGATGACGATCATGTGTTTC	Exon5	375 bp
ECSOD-SNP-R5	ACAACTCTTGACGTCTTGACCATCT		