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Identification of a Serum amyloid A gene and the association of SNPs with *Vibrio*-resistance and growth traits in the clam *Meretrix meretrix*

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ABSTRACT

Serum amyloid A (SAA), an acute response protein as well as an apolipoprotein, is considered to play crucial roles in both innate immunity and lipid metabolism. In this study, a *SAA* gene (*MmSAA*) was identified in the clam *Meretrix meretrix*. The full length DNA of *MmSAA* was 1407 bp, consisting of three exons and two introns. The distribution of *MmSAA* in clam tissues was examined with the highest expression in hepatopancreas. In response to the *Vibrio parahaemolyticus* challenge, *MmSAA* mRNA showed significantly higher expression at 24 h post-challenge in experimental clams ($P < 0.05$). Forty-eight single nucleotide polymorphisms (SNPs) in the DNA partial sequence of *MmSAA* were discovered and examined for their association with *Vibrio*-resistance and growth traits, respectively. The single SNP association analysis indicated that five single SNPs (g.42, g.72, g.82, g.147 and g.165) were significantly associated with *Vibrio*-resistance ($P < 0.05$). Haplotype analysis produced additional support for association with the Chi-square values 6.393 ($P = 0.012$). Among the five selected SNPs, the effect of a missense mutation (g.82, A → G) was detected by site-directed mutagenesis with fusion expression of protein assay, and the result showed that the recombinant plasmids containing wild-type pET30a-MmSAA had more inhibition effect than the mutant ones on the growth rate of the host bacteria. In addition, four growth traits of the clams in 09G3SPSB population were recorded and the SNP g.176 was found to be significantly associated with the growth traits with the Global score value 0.790 ($P = 0.015$). Our findings suggested that common genetic variation in *MmSAA* might contribute to the risk of susceptibility to *Vibrio* infection and might be associated with the growth traits in the clams *M. meretrix*, and more works are still needed to validate these SNPs as potential markers for actual selective breeding.

1. Introduction

The clam *Meretrix meretrix* is an important economic species of marine bivalve, mainly distributed in the shallow seas of South and Southeast Asia [1]. With the success of artificial breeding, *M. meretrix* has become one of the widely cultured bivalves in China [2]. In recent years, clam diseases caused by bacteria and virus are becoming more and more common and serious, which inflicted huge financial losses [3]. Hence, it is urgent and crucial to carry out genetic breeding for the selection of high *Vibrio*-resistance strains of the clam *M. meretrix*.

Marker-assisted selection (MAS) is a powerful method with which breeders can select animals with desirable combination of genes. Particularly, previous studies have shown that the genetic improvement is more significant and effective for less domesticated aquatic animals than terrestrial farm animals through MAS methods [4]. Up to now, several successful cases have been reported in breeding programs for disease control and fast-growing in several species of fishes and protostome invertebrates with MAS method [5–9]. Screening of molecular markers associated with certain traits is the first step for MAS [10,11]. Among the molecular markers, single nucleotide polymorphisms (SNPs) are co-dominant, biallelic and distributed widely with high density. Up to now, discovery of SNPs by candidate gene approach is still one of the efficient ways for non-model organisms [12,13].

Serum amyloid A (SAA), one of the main acute proteins in vertebrates, has been reported involving the animals innate immune [14–17], which is often observed to increase markedly within the first 48 h after the triggering event and has a rapid decline due to their short half-life [18,19]. Further study showed that SAA can bind

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to many Gram-negative bacteria through outer membrane protein A (OmpA) family members [20]. And at the same time, it is involved in up-regulating PMN antimicrobial activities and yet high circulating concentrations of SAA as seen in the acute phase responses may constitute a potential host defense mechanism against fungal infection [21]. Besides of its immune roles, SAA is found to be expressed in human adipose tissue during the non-acute-phase reaction condition [22–24]. As apolipoprotein, SAA is determined to be able to affect lipid metabolism through regulation of the expression of multiple genes related to lipid metabolism [19,25–27]. Recent reports indicated that SAA is a growth factor for 3T3-L1 adipocytes, which can inhibit differentiation and promote insulin response [28]. Though *SAA*, a highly conserved gene in mammals, has been reported in many vertebrates, the first and the only article about *SAA* in mollusc was published in 2014 which suggest that *Ch*SAA is likely to constitute a member of the A-SAA family involved in anti-pathogen responses in oyster *Crassostrea hongkongensis* [29].

In the present study, we cloned the cDNA sequence of *SAA* (*MmSAA*) and detected its tissue distribution in *M. meretrix*. The mRNA variation of *MmSAA* was also analyzed after the clams challenged by *Vibrio parahaemolyticus*. Furthermore, considering the possible roles of SAA in immune system and lipid metabolism, we choose *MmSAA* as a candidate gene to detect SNPs associated with *Vibrio*-resistance and growth traits in the clam *M. meretrix*. The recombinant plasmids of pET30a-MmSAA (wild-type and mutant) were constructed and the effect were tested by comparing the growth rate of the host bacteria. To our knowledge, this study was the first time to identify a *SAA* gene and analyze SNPs associated with resistance and growth traits in clams. We hope the results would be helpful for genetic selection of the excellent varieties which have the features of fast-growing and/or high-resistance against pathogens in clam *M. meretrix*.

2. Materials and methods

2.1. Experimental clams

The clams (46.51 ± 0.32 mm in shell length) from Shandong population were bought from aquatic market in Qingdao, China. Twenty calms were selected for RNA isolation, DNA isolation, cDNA synthesis and DNA synthesis, and then used for the detection of SNPs distribution in the DNA sequence of *MmSAA*. One hundred clams were acclimated for one week in our laboratory and then used for the challenge experiment. Clams were maintained at 25–26 °C, fed with condensed microalgae and kept in continuous aeration during the acclimation period.

Clam samples from three groups (11-C, 11-S and 11-R) [30,31] were applied to evaluate the SNP makers and identify SNPs associated with *Vibrio*-resistance. In brief, in year 2011, 50 clams randomly collected from Shandong natural population formed the control group (11-C) to evaluate the candidate SNP markers. At the same time, about 1000 clams from the same population were challenged with *V. parahaemolyticus*, the clams from which were divided into the *Vibrio*-resistant group (11-R) and the susceptive group (11-S) according to their survived time.

An independent clam population with significant growth variances (09G3SPSB) [32], generated in the year 2009, was used to identify SNPs associated with the growth traits (shell length, shell width, shell height, and shell weight).

2.2. Vibrio challenge and sample collection

Before the challenge test, five tissues (hepatopancreas, mantle, foot, gill and adductor muscle) were collected from four healthy clams for the tissue distribution analysis. Sixty clams after acclimation were randomly separated into *Vibrio*-challenged and control groups. A *V. parahaemolyticus* strain (MM21), isolated from clams and characterized to be pathogenic to *M. meretrix* [3], was used to the challenge test. Briefly, 30 clams were injected with 50 μl–5 × 10^6 CFU ml^{-1} of MM21 in PBS (MM21-injected group) and 30 clams were injected with 50 μl PBS (control group). Then the clams were reared in tanks and sampled at the 0 h, 6 h, 12 h, 24 h and 48 h post-challenge, respectively. The hepatopancreas were dissected and preserved in liquid nitrogen for RNA extraction. There were four replicates for each time point.

2.3. RNA and DNA extraction and cDNA synthesis

RNA of the different samples were extracted by Unizol Total RNA Isolation Reagent (Promega, USA) according to the manufacturer's protocol. cDNA was systhesized from total RNA with M-MLV reverse transcriptase (Promega, USA), oligo (dT) primer and BDA oligo according to the manufacturer's protocol of SMART RACE cDNA Amplification Kit (Clontech, USA).

Totol genomic DNA (gDNA) was extracted from foot tissue of each clam using DNA extraction kit special for marine animals (Tiangen, China) following the manufacturer's protocol. DNA stocks were diluted to 100 ng/μl and used as templates in PCR reactions for sequencing.

2.4. Cloning of full length cDNA and DNA of MmSAA

The full-length cDNA sequence of *MmSAA* was obtained by 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) using SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Cloning primers of MmSAA5′GSP, MmSAA5′NGSP, MmSAA3′GSP and MmSAA3′NGSP were designed according to the contig21074 which is similar to *SAA* genes from the transcriptome sequencing data of clam *M. meretrix* analyzed using the NGS [33]. The PCR products were purified, inserted into the pMD19-T vector using TA cloning kit (TakaRa), respectively. Clones with confirmed recombinant plasmids were sequenced by Beijing Genomics Institute (BGI). The full-length cDNA of *MmSAA* was assembled by alignment of the partial cDNA fragment, 5′- and 3′-RACE fragments with the aid of SeqMan program of Larsergene software. In order to confirm the full length cDNA of *MmSAA*, a pair of primers (cMmSAAF and

Table 1
PCR primers in this study.

Primers	Sequences (5′–3′)	Usage
MmSAA5′GSP	CAATCGCCCGTTGAGACCAT	5′-Race PCR
MmSAA5′NGSP	AGTTCCCCTCTGTGCTGCCT	5′-Race PCR
MmSAA3′GSP	CGTGGTCTTGACAGGCGTTACTA	3′-Race PCR
MmSAA3′NGSP	TACTTACTTCACTAAAGCACAGGACACT	3′-Race PCR
cMmSAAF	CAGAACATACAGAGCGATGAAGC	Sequence verify and SNP genotyping
cMmSAAR	ATTTAGTGTCCTGTGCTTTAGTGAA	Sequence verify and SNP genotyping
MmSAA-RT-F	GACGCTGACCAAGAAGCCAAC	Quantitative RT-PCR
MmSAA-RT-R	GAAGCATGTCTTAGTAACGCCTGTC	Quantitative RT-PCR
actin-F	TTGTCTGGTGGTTCAACTATG	Quantitative RT-PCR
actin-R	TCCACATCTGCTGGAAGGTG	Quantitative RT-PCR
ESAA-F	***CGGGATCC***AACGTTTTCCAACGA GCT***A***GGA	Point mutation and prokaryotic expression
ESAA-F′	***CGGGATCC***AACGTTTTCCAACGA GCT***G***GGA	Point mutation and prokaryotic expression
ESAA-R	***ATAAGAATGCGGCCGC***GTAACG CCTGTCAAGACCACG	Point mutation and prokaryotic expression

The positions of SNP g.82 were shown in bold and italics letters. Tails added to primers are shown in bold, italic letters.