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# Identification of agmatinase from *Vibrio splendidus* and its roles in modulating arginine metabolism of *Apostichopus japonicus*

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ABSTRACT

Innate immunity is the first line of defense against invading pathogens. However, pathogenic bacteria have developed various mechanisms, such as direct competition for common substrates and transformation of metabolic regulations of the host, which allow them to escape from the immune surveillance system and enhance their survival. In this study, the *Vibrio splendidus* agmatinase (designated as *Vsagmatinase*) gene was cloned, and its biological activities in *Apostichopus japonicus* coelomocytes were analyzed. Vsagmatinase encoded an open reading frame of 930 bp, which translated into a predicted protein of 309 amino acid residues. SMART analysis and multiple sequence alignment indicated that Vsagmatinase displayed an arginase domain, and the conserved histidine ($H^{151}$ and $H^{163}$) and aspartic acid ($D^{125}$, $D^{149}$, $D^{153}$, $D^{231}$, and $D^{233}$) residues for catalytic activity were all strictly conserved in this domain. Moreover, four conserved residues, namely, $Asp^{153}$, $His^{163}$, $Thr^{245}$, and $Glu^{275}$, that bonded with the guanidinium portion of agmatine were conserved in *V. splendidus* agmatinase. The recombinant protein was expressed in *Escherichia coli* (BL21), and the purified Vsagmatinase showed high agmatinase activity with agmatine substrate. By site-directed mutagenesis, the agmatinase activity of mutated Vsagmatinase was reduced to approximately 8.9%–9.4% of wild-type activity under different Vsagmatinase protein concentrations, indicating that the $Glu^{275}$ residue was necessary for the guanidinium group of agmatine. Notably, injection of Vsagmatinase in coelomocytes could significantly increase both the mRNA expression levels and enzyme activities of *Ajarginase* and *Ajagmatinase* and obviously inhibited *AjNOS* transcript and NO content in *A. japonicus*. By contrast, mutated Vsagmatinase downregulated the activity of arginase and promoted NO production at 12 h after injection. Our findings suggested that Vsagmatinase served as the competitor that utilizes host agmatine and modulates arginine metabolism in *A. japonicus*.

## 1. Introduction

Agmatinase (agmatine ureohydrolase, EC 3.5.3.11) catalyzes the irreversible hydrolysis of the guanidinium group of the agmatine side chain to form putrescine and urea (Satishchandran and Boyle, 1986). Agmatine generated from decarboxylation of L-arginine by arginine decarboxylase (ADC, EC 4.1.1.19) is a metabolic intermediate in the biosynthesis of polyamines (including putrescine, spermidine, and spermine). It also exhibits important regulatory functions in mammals (Buch and Boyle, 1985; Satishchandran and Boyle, 1986; Iyer et al., 2002). In general, the classic mammalian pathway for polyamine biosynthesis proceeds from L-arginine by the enzyme of arginase, which also competes with nitric oxide synthase (NOS) by using the common substrate of L-arginine during pathogen invasion (Popovic et al., 2007; Wanasen and Soong, 2008).

The metabolism of L-arginine to polyamines via agmatinase is an alternative pathway long recognized in lower organisms (Wang et al., 2014). Nowadays, agmatinase has been obtained and described in many eukaryotes (Vicente and Legaz, 1982; Shimotohno et al., 2003), but the best characterized enzymes are from *Escherichia coli* and *Deinococcus radiodurans* (Romero et al., 2017). Agmatinase and arginase are both binuclear manganese metalloenzymes and belong to the ureohydrolase superfamily (Perozich et al., 1998). The deduced amino acid sequences of agmatinases have high homology to all sequenced arginases (Perozich et al., 1998; Iyer et al., 2002; Goda et al., 2008), which also catalyze a hydrolytic reaction with the production of urea. The two enzymes both exhibit an absolute requirement of $Mn^{2+}$ for catalytic activity (Kanyo et al., 1996; Carvajal et al., 1999a), and the seven conserved residues (histidine and aspartic acid) essential for metal binding and substrate hydrolysis for arginases are highly conserved in agmatinases (Kanyo et al., 1996; Carvajal et al., 1999a,b; Mistry et al., 2002; Salas et al., 2004; Romero et al., 2017).

Although agmatinase and arginase use different substrates for catalyzing hydrolytic reaction, they both generate urea and polyamines.

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Recent studies supposed that different pathogens, such as viruses and bacteria, can regulate host agmatinase and arginase at the mRNA and protein levels, thereby inducing polyamine synthesis (Mistry et al., 2002; Benedicenti et al., 2017). An important characteristic of polyamines is to increase the cell growth and proliferation, especially bacterial and parasitic pathogen colonization in host, which are harmful to host tissues and cells (Byers et al., 1991; Ghosh et al., 2009). When the arginine metabolic pathway is controlled by arginase, polyamine biosynthesis will be generated and will decrease the NO level, thereby favoring pathogen growth (Iniesta et al., 2002). More importantly, bacterial arginase can directly use host substrate to generate urea and the conversion of arginine to polyamines and urea via the arginase pathway can support the growth of pathogens (Das et al., 2010). Borlace et al. (2012) found that *Helicobacter pylori* can use its own arginase (RocF) to escape the bactericidal effects of macrophage-derived NO through competition using the common substrate L-arginine from the host. In contrast to arginase, whether bacterial agmatinase directly uses host agmatine has not yet been elucidated.

The invertebrate sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea) with an innate immune system is one of the commercially important species in Chinese marine culture (Han et al., 2016). However, it is susceptible to bacterial infection, leading to disease outbreaks (Deng et al., 2009; Liu et al., 2010). The main pathogens include *Vibrio* (Ma et al., 2006) and *Pseudomonas* (Ma et al., 2006) species, wherein *Vibrio splendidus* is widely accepted as one of the major pathogens that infects *A. japonicus* (Zhang et al., 2006). To date, only few studies are available on the pathogenic mechanism of *V. splendidus* in infecting sea cucumbers (Liang et al., 2016). In our previous study, we found that *A. japonicus* arginase and agmatinase cooperatively compete with NOS for arginine utilization under *V. splendidus* infection (Shao et al., 2016). When we screened for the genomic sequence of *V. splendidus*, we found only *agmatinase*. The importance of the *agmatinase* gene to *V. splendidus* whether function as *arginase* is pathogenic to host should be explored. Therefore, this study aims to explore the role of *agmatinase* in *V. splendidus* and identify whether bacterial *agmatinase* gene is involved in the innate immunity of *A. japonicus*.

## 2. Materials and methods

### 2.1. Ethical statement

Sea cucumbers (*A. japonicus*) used here were commercially cultured animals. All experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The study protocol was approved through the Experimental Animal Ethics Committee of Ningbo University, China.

### 2.2. Bacterial strains, experimental animals, and culture conditions

*V. splendidus* was isolated from sea cucumbers suffering from skin ulceration syndrome collecting from indoor farms of Jinzhou Hatchery in May 2013, and its identity was determined by 16S rDNA sequencing analysis. The bacteria was preserved in glycerol and stocked at −80 °C for further utilization. The bacteria were cultured in liquid 2216E broth (tryptone 5 g/L, yeast extract 1 g/L, pH 7.6) at 28 °C.

Healthy adult *A. japonicus* with average wet weight of 125 ± 15 g were collected from Dalian Pacific Aquaculture Company (Dalian, China) and quarantined in aerated natural seawater with salinity of 28‰ at 16 °C for at least three days for challenge experiments.

### 2.3. DNA manipulation, plasmid construction, and site-directed mutagenesis

For *V. splendidus* plasmid preparation, DNA fragments were extracted using Bacterial DNA Kit (OMEGA) according to the manufacturer's instructions. The primers used in this study are listed in Table 1. Primers were designed according to the nucleotide sequence of gene encoding agmatinase from the genomic DNA of *V. splendidus* strain LGP32, with accession number FM954973. PCR amplifications were carried out with a Biometra PCR system (Biometra, Germany). To obtain recombinant Vsagmatinase, second round PCR was carried out using primers with specific restriction sites (Table 1) and using fragment from the first round PCR as template. The PCR products were cloned into the pMD19-T simple vector (TaKaRa), digested with *EcoR* I (Thermo) and *Not* I (Thermo), and inserted into *EcoR* I and *Not* I digested pET-28a (+) expression vector (Novagen) (termed pET-28a-Vsagmatinase-WT). The plasmid was sequenced to ensure correct construction, and plasmid DNA for site-directed mutagenesis was purified with an E.Z.N.A.™ Plasmid Mini Kit (OMEGA). To test the possible function of conserved $Glu^{275}$ residue of pET-28a-Vsagmatinase-WT, a mutant was generated using the Fast Mutagenesis System kit (*TransGen*) according to the manufacturer's instructions. The expected mutation and absence of unwanted mutations were confirmed by sequence analysis. Both recombinant plasmids (pET-28a-Vsagmatinase-WT/MT) were further transformed into *E. coli* BL21 (DE3) (Novagen) and subjected to DNA sequencing again. The positive clones were subsequently incubated in LB medium containing 50 mg/mL of kanamycin at 37 °C and 200 rpm and induced by the addition of 1 mM of isopropyl-b-D-thiogalactopyranoside (IPTG) with normal cell growth.

**Table 1**
Primers used in this study.

| Primer name | Primer sequence (5-3′) | Used for |
|---|---|---|
| *Vsagmatinase* F | ATGAACGATCTATTTACGAAACC | ORF amplification |
| *Vsagmatinase* R | TTATGATCTAGCTAGACGATTCG | |
| *Vsagmatinase EcoRI* F | GAATTCATGAACGATCTATTTACGAAACC | Vector construction |
| *Vsagmatinase NotI* R | GCGGCCGCTTATGATCTAGCTAGACGATTCG | |
| *Vsagmatinase* MF | GTATGGACGTTGTAGCAGTGTCTCCA | Site-directed mutagenesis |
| *Vsagmatinase* MR | GCTACAACGTCCATACCAACCATGTT | |
| *AjNOS* F | GTAGAAGGAAAGGAGAGCGAGTC | Real-time PCR |
| *AjNOS* R | GTAGAAGGAAAGGAGAGCGAGTC | |
| *Ajarginase* F | AAGCGTTGGGATTCTCGGTGTG | |
| *Ajarginase* R | TGGGAGTTCTTCACGAGAGGTTG | |
| *Ajagmatinase* F | CCTTACGAGTTGCCGACATTGGT | |
| *Ajagmatinase* R | CTCGTCAAATGCCCTACGGAATG | |
| *Ajβ-actin* F | CCATTCAACCCTAAAGCCAACA | |
| *Ajβ-actin* R | ACACACCGTCTCCTGAGTCCAT | |

### 2.4. Sequence analysis

Sequences homology were obtained using BLAST program at National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast) and the deduced amino acid sequence of Vsagmatinase was analyzed with the expert protein analysis system (http://www.expasy.org/). Domain in amino acid sequence was detected using the simple modular architecture research tool (SMART) program (http://www.smart.emblheidelbergde/) and multiple alignments analysis of each protein were performed using the ClustalW2 multiple alignment program (http://www.ebi.ac.uk/clustalw/). The signal peptide, protein domain features and trans-membrane domain were predicted by Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 program.