Short communication

Metabolic profiling of the tissue-specific responses in mussel *Mytilus galloprovincialis* towards *Vibrio harveyi* challenge

Xiaoli Liu a,*, Chenglong Ji b, Jianmin Zhao b, Qing Wang b, Fei Li b, Huifeng Wu b

a School of Life Sciences, Ludong University, Yantai 264025, PR China

b Laboratory of Coastal Ecotoxicology, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, PR China

**A R T I C L E   I N F O**

Article history:
Recieved 22 January 2014
Received in revised form 7 April 2014
Accepted 27 May 2014
Available online 6 June 2014

Keywords:
Metabolomics
*M. galloprovincialis*
Digestive gland
Gill
*V. harveyi*

**A B S T R A C T**

Mussel *Mytilus galloprovincialis* is a marine aquaculture shellfish distributing widely along the coast in north China. In this work, we studied the differential metabolic responses induced by *Vibrio harveyi* in digestive gland and gill tissues from *M. galloprovincialis* using NMR-based metabolomics. The differential metabolic responses in the two tissue types were detected, except the similarly altered taurine and betaine. These metabolic responses suggested that *V. harveyi* mainly induced osmotic disruption and reduced energy demand via the metabolic pathways of glucose synthesis and ATP/AMP conversion in mussel digestive gland. In mussel gill tissues, *V. harveyi* basically caused osmotic stress and possible reduced energy demand as shown by the elevated phosphocholine that is involved in one of the metabolic pathways of ATP synthesis from ADP and phosphocholine. The altered mRNA expression levels of related genes (superoxide dismutase with copper and zinc, heat shock protein 90, defensin and lysozyme) suggested that *V. harveyi* induced clear oxidative and immune stresses in both digestive gland and gill tissues. However, the mRNA expression levels of both lysozyme and defensin in digestive gland were more significantly up-regulated than those in gill from *V. harveyi*-challenged mussel *M. galloprovincialis*, meaning that the immune organ, digestive gland, was more sensitive than gill. Overall, our results indicated that *V. harveyi* could induce tissue-specific metabolic responses in mussel *M. galloprovincialis*.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Vibrios, such as *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio splendidus*, *Vibrio furnissii*, *Vibrio para-haemolyticus*, *Vibrio tapetis* and *Vibrio harveyi*, are widely distributed in marine environments and main causative pathogens of vibrosis in both fish and shellfish [1,2]. As a type of vibrios, *V. harveyi* is a gram-negative bacterium and its outbreak is a causative agent for the massive mortality of marine aquaculture animals due to its high accumulation and susceptibility to pathogens [7–9]. For example, Canesi et al. found that infections of vibrios (*V. splendidus* and *V. anguillarum*) induced oxidative and immune stresses in *M. galloprovincialis*, indicated by significant up-regulations of gene expression levels of immune stress-responsive molecules, including anti-oxidant enzymes (catalase and glutathione transferase), lysozymes and metallothioneins [8].

In many previous immunity studies, researchers focused on the special immune-related molecules and their functions involved in immune network in *M. galloprovincialis* [7–10]. With the development of system biology techniques (genomics, transcriptomics, proteomics and metabolomics), researchers have applied these -omic approaches to fish and shellfish immunology, which can provide a comprehensive understanding of immune responses of marine aquaculture animals to pathogen challenges [11,12]. Recently, Ji et al. reported the proteomic responses in *M. galloprovincialis* challenged by another vibrio, *V. anguillarum* [12]. They found that the infection of *V. anguillarum* caused disruption in energy metabolism and oxidative stress in *M. galloprovincialis*, indicated by the proteomic biomarkers, including procollagen-proline dioxygenase, protein disulfide...
isomerase, nucleoside diphosphate kinases, electron transfer flavo-protein and glutathione S-transferase [12]. In our previous work, we confirmed that V. anguillarum caused more severe oxidative and immune stresses in the digestive gland from clam Ruditapes philippinarum than V. splendidus using NMR-based metabolomics [13].

As an immune organ, the digestive gland of marine bivalves is the most frequently used organ in immunity studies due to the filter-feeding habit of M. galloprovincialis [8,11,13]. The organ of Gill can be directly attacked by marine pathogens and has been also investigated in shellfish immunology [12,14,15]. In this work, the metabolic profiles were profiled in both digestive gland and gill tissues from mussel M. galloprovincialis challenged by V. harveyi. The aim of this work was to illustrate the differential metabolic effects and mechanisms induced by V. harveyi in digestive gland and gill tissues of M. galloprovincialis using NMR-based metabolomics.

2. Materials and methods

2.1. Animals and experimental conditions

Adult mussels M. galloprovincialis (shell length: 5.0–6.0 cm, n = 45) were collected in July 2013 from an aquaculture farm (Yantai, China). Animals were transported to the laboratory and acclimatized in aerated natural seawater (salinity 31.1 psu) for 10 d. After acclimatization, the animals were randomly divided into three groups (blank, PBS control and V. harveyi challenge) each containing 15 individuals in 30 L filtered seawater. The culture seawater was renewed daily. During the acclimation period, animals were kept at 25 °C under a photoperiod of 12 h light and 12 h dark, and fed with the Chlorella vulgaris at a ration of 2% tissue dry weight daily.

2.2. Challenge experiment

The bacterium, V. harveyi, was kindly provided by Prof. Baozhong Liu (Institute of Oceanology, CAS). The bacteria were cultured in liquid 2216E broth (Trypsitone 5 g L⁻¹, yeast extract 1 g L⁻¹, CaH₂Fe·5H₂O 0.1 g L⁻¹, pH 7.6) at 29 °C and centrifuged at 3000 g for 5 min to harvest the bacteria. For challenge experiment, live bacteria of V. harveyi were re-suspended in PBS. Each challenged mussel was injected with V. harveyi (10⁵ CFU/mL) in 50 μL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ 2 mM KH₂PO₄ pH 7.4) in the adductor muscle. As a control, the mussel tissue (100 mg) was homogenized in 400 μL of methanol and 85 μL of water. Then the mixture was shaken and centrifuged (5 min, 3000 g, at 4 °C), and the supernatant substance was transferred into a glass vial. A total of 200 μL of chloroform and 440 μL of water were added to the supernatant, and the mixture was vortexed and centrifuged again (10 min, 3000 g, 4 °C). The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts of tissues were then re-suspended in 600 μL of phosphate buffer (100 mM Na₂HPO₄ and NaH₂PO₄, including 0.5 mM 2,2,3,3-d(4)-3-(trimethylsilyl)propionic acid sodium salt (TSP) as the internal standard, pH 7.0) in D₂O. The mixture was vortexed and centrifuged at 3000 g for 5 min at 4 °C. The supernatant substance (550 μL) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

2.4. RNA extraction and quantitation of gene expressions

Total RNA from mussel digestive gland and gill tissues was extracted following the manufacturer's directions (Invitrogen, LifeTechnologies, Carlsbad, CA, USA), and the first-strand cDNA was synthesized according to M-MLV RT Usage information (Promega, Madison, WI, USA). The expressions of the housekeeping genes in M. galloprovincialis were determined by real-time quantitative PCR (qRT-PCR), the data were analyzed with geNorm to calculate the expression stability of the genes (M values) and the optimal number of reference genes required for accurate normalization (V values) [19]. GeNorm identified β-actin as the most stable gene which was then used as the internal control for gene expression normalization (data not shown).

Gene-specific primers (Table 1) for selected genes were used to amplify amplicons specific for M. galloprovincialis. The fluorescent qRT-PCR amplifications were carried out in triplicate in a total volume of 20 μL containing 10 μL of 2 × SYBR Premix Ex Taq™ (TaKaRa), 0.4 μL of 50 × ROX Reference DYE II, 4.8 μL DEPC-treated H₂O, 0.4 μL of each primer, 4.0 μL of 1:20 diluted cDNA. The qRT-PCR program was as following: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 45 s, 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the ABI 7500 SD software (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The comparative CT method (2⁻ΔΔCT method) was used to analyze the expression level of the genes [20]. One-way ANOVA combined with Tukey's test was performed on mRNA expression levels between PBS control and V. harveyi-challenged groups, respectively. A P value less than 0.05 was considered statistically significant. The Minitab software (Version 15, Minitab Inc., USA) was used for the statistical analysis.

2.5. ¹H NMR spectroscopy

Metabolite extracts of tissues from mussels were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at
25 °C as described previously [21]. All 1H NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

2.6. Spectral pre-processing and multivariate data analysis

All one dimensional 1H NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathWorks, Natick, MA, USA) [22]. Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed with a transformation parameter $\lambda = 2.0 \times 10^{-3}$ to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks [22]. Data were mean-centered before multivariate data analysis.

The supervised multivariate data analysis methods, partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structure with discriminant analysis (O-PLS-DA), were sequentially carried out to uncover and extract the statistically significant metabolite variations related to V. harveyi challenges. The results were visualized in terms of scores plots to show the classifications and corresponding loadings plots to show the NMR spectral variables contributing to the classifications. The model coefficients were calculated from the coefficients incorporating the weight of the variables in order to enhance interpretability of the model and the value of $Q^2$ was used to describe the predictive ability of the constructed model and assess the model quality. Then metabolic differences responsible for the classifications between control and V. harveyi-challenged groups could be detected in the coefficient-coded loadings plots. The details regarding data analysis were described previously [23]. Metabolites were assigned following the tabulated chemical shifts [24] and the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada).

3. Results and discussion

3.1. Metabolic differences in digestive gland and gill tissues from M. galloprovincialis from PBS control group

As a system biology technique, metabolomics has been widely employed in multiple areas, including toxicology and immunology [11,13,25–30]. Fig. 1 indicates the 1H NMR spectra of tissue extracts of digestive gland (Fig. 1A) and gill (Fig. 1B) samples from PBS control mussel group. The identified metabolites, including amino acids (branched chain amino acids: valine, leucine and isoleucine, aspartate, glutamate, glutamine, tyrosine phenylalanine, etc.), energy storage compounds (ATP, glucose and glycogen), an intermediate in Krebs cycle (succinate), osmylates (betaine, taurine, glycine and homarine) and a phosphagen (phosphocholine), were labeled in Fig. 1. As shown in Fig. 1, both 1H NMR spectra are dominated by the organic osmylate, betaine (3.27 and 3.90 ppm), which is synthesized from a two-step reaction of choline: choline → betaine aldehyde + NAD$^+$ → betaine + NADH [31]. Since digestive gland is the main organ of glucose and protein metabolisms, the 1H NMR spectrum from the digestive gland sample should present the higher levels of most amino acids and glucose than those in the 1H NMR spectrum from the gill sample. Initially, O-PLS-DA was performed on the NMR spectral datasets from the blank and PBS control groups. However, no significant separation between blank and PBS groups was found (data not shown), which suggested that the single injection of PBS in the adductor muscle did not obviously affect the metabolic profiles in both digestive gland and gill tissues. Therefore, only PBS control was used in further data analysis. O-PLS-DA analysis resulted in a clear separation between the digestive gland and gill samples from PBS control group (Fig. 2A), with a $Q^2$ value greater than 0.9 showing the robustness of the constructed model, which confirmed the significant metabolic differences between these two tissues. Clearly, the corresponding loading plot (Fig. 2B) displayed the higher levels of most amino acids (threonine, alanine, glutamate, glutamine, etc.) and glucose, as well as the higher levels of taurine and homarine and lower levels of aspartate and betaine in digestive glands than those in gills. These biological (metabolic) differences between digestive gland and gill tissues suggested the potential differential metabolic responses in these two organs towards V. harveyi challenge.

3.2. Differential metabolic responses in digestive gland and gill tissues from M. galloprovincialis challenged by V. harveyi

O-PLS-DA was conducted on the 1H NMR spectral data to characterize the metabolic responses in digestive gland and gill tissue samples from PBS control group, respectively (Fig. 3). Fig. 3A and C indicated that O-PLS-DA resulted in clear classifications between PBS control and V. harveyi-challenged mussel groups, respectively, with reliable $Q^2$ values (>0.7). From the loading plot (Fig. 3B), the concentrations of adenosine and AMP were significantly ($P < 0.05$) increased in V. harveyi-challenged mussel digestive gland. The
levels of aspartate, dimethylglycine, glucose, ATP, taurine and betaine were significantly ($P < 0.05$) decreased. Adenosine plays important roles in both energy transfer as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), as well as in signal transduction as cyclic adenosine monophosphate [32]. In the digestive gland from V. harveyi-challenged mussels, the depleted ATP meant a reduced energy demand resulting in the elevated organic osmolytes, including hypotaurine, homarine and glycine, clearly indicated the osmotic stress caused by V. harveyi challenge in the gill of mussel M. galloprovincialis. However, other two osmolytes, taurine and betaine were depleted. Since taurine can synthesized into hypotaurine, the decrease of taurine meant the enhanced synthesis of hypotaurine from taurine, resulting in an increase of hypotaurine in V. harveyi-challenged mussel gill samples. Similarly, betaine is synthesized from glycine. Therefore, the depletion of betaine combined with the elevation of glycine indicated the reduced synthesis of betaine in V. harveyi-challenged mussel gill samples. Ji et al. reported the similar metabolic responses including depleted taurine and betaine and elevated homarine in gill tissues from mussel M. galloprovincialis challenged by V. anguillarum, which implied the similar disturbance in osmotic regulation induced by both V. anguillarum and V. harveyi in mussel gills [12]. As a phosphagen, phosphocholine is involved in the conversion of ATP and choline into phosphocholine and ADP, which is catalyzed by choline kinase. Therefore, the elevated phosphocholine probably meant a reduced energy demand in V. harveyi-challenged mussel gills via a different metabolic pathway compared with that in digestive gland, as mentioned above.

3.3. Stress-responsive gene expressions in digestive gland and gills from M. galloprovincialis challenged by V. harveyi

In this study, four stress-responsive genes including heat shock protein 90 (HSP90), superoxide dismutase with copper and zinc (Cu/Zn-SOD), defensin and goose-type lysozyme related to diverse functions were used for the quantification of mRNA expressions. HSP90, Cu/Zn-SOD, defensin and lysozyme play important roles in anti-oxidative stress and immune defense. After V. harveyi injection, the mRNA expression levels of HSP90, Cu/Zn-SOD, defensin and lysozyme mRNA in both digestive gland and gill tissues were quantified using qRT-PCR technique with $\beta$-actin as internal control (Fig. 4). All the mRNA expression levels of these four selected stress-responsive genes were significantly ($P < 0.05$) up-regulated in V. harveyi-challenged mussel digestive gland and gill tissues (Fig. 4). Cu/Zn-SOD is a known anti-oxidant enzyme involved in scavenging reactive oxygen species and HSP90 belongs to a large family of molecular chaperones playing vital roles in preventing irreversible protein denaturation, aggregation and misfolding [38,39]. They can be induced by oxidative stress and pathogen infection [38]. Both
defensin and lysozyme are antibacterial components that have been characterized in marine mollusks [40,41]. In this work, the significant up-regulation of mRNA expression levels of Cu/Zn-SOD, HSP90, defensin and lysozyme indicated the oxidative and immune stresses induced by V. harveyi in both digestive gland and gill samples from V. harveyi-challenged mussels. Consequently, metabolites that are more abundant in the control group are presented as peaks in the negative direction. Keys: (1) aspartate, (2) dimethylglycine, (3) taurine, (4) betaine, (5) adenosine, (6) β-glucose, (7) α-glucose, (8) AMP, (9) ATP, (10) unknown 1 (1.10 ppm), (11) phosphocholine, (12) hypotaurine, (13) glycine and (14) homarine.

In summary, the differential metabolic responses induced by V. harveyi in digestive gland and gill tissues from M. galloprovincialis.
were investigated using NMR-based metabolomics. Overall, V. harveyi induced osmotic disruption and reduced energy demand via the metabolic pathways of glucose synthesis and ATP/AMP conversion in mussel digestive gland. In mussel gill tissues, V. harveyi basically caused osmotic stress and possible reduced energy demand through the metabolic pathway of conversion of phosphocholine and ADP to choline and ATP. The altered mRNA expression levels of related genes (Cu/Zn-SOD, HSP90, defensin and lysozyme) suggested that V. harveyi induced clear oxidative and immune stresses in both digestive gland and gill tissues. However, the mRNA expression levels of both lysozyme and defensin in digestive gland were more significantly up-regulated than those in gill from V. harveyi-challenged mussel M. galloprovincialis. This work demonstrated that V. harveyi could induce tissue-specific metabolic responses in the digestive gland and gill tissues from mussel M. galloprovincialis using NMR-based metabolomics.

Acknowledgment

This work was supported by A Project of Shandong Province Higher Educational Science and Technology Program (No. J14LE08). The authors thank Prof. Mark Viant for the use of ProMeta software.

References