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Divergent Metabolic Responses of *Apostichopus japonicus* Suffered from Skin Ulceration Syndrome and Pathogen Challenge

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ABSTRACT: Skin ulceration syndrome (SUS) is the main limitation in the development of *Apostichopus japonicus* culture industries, in which *Vibrio splendidus* has been well documented as one of the major pathogens. However, the intrinsic mechanisms toward pathogen challenge and disease outbreak remain largely unknown at the metabolic level. In this work, the metabolic responses were investigated in muscles of sea cucumber among natural SUS-diseased and *V. splendidus*-challenged samples. The pathogen did not induce obvious biological effects in *A. japonicus* samples after infection for the first 24 h. An enhanced energy storage (or reduced energy demand) and immune responses were observed in *V. splendidus*-challenged *A. japonicus* samples at 48 h, as marked by increased glucose and branched chain amino acids, respectively. Afterward, infection of *V. splendidus* induced significant increases in energy demand in *A. japonicus* samples at both 72 and 96 h, confirmed by decreased glucose and glycogen, and increased ATP. Surprisingly, high levels of glycogen and glucose and low levels of threonine, alanine, arginine, glutamate, glutamine, taurine and ATP were founded in natural SUS-diseased sea cucumber. Our present results provided essential metabolic information about host−pathogen interaction for sea cucumber, and informed that the metabolic biomarkers induced by *V. splendidus* were not usable for the prediction of SUS disease in practice.

KEYWORDS: *Apostichopus japonicus, muscles, metabolomics, skin ulceration syndrome, Vibrio splendidus*

INTRODUCTION

Sea cucumber *Apostichopus japonicus* (Echinodermata, Holothuroidea), an important marine animal for commercial fisheries, is widely distributed in the coast of north China and has become one of the predominant industries accompanied by its rapid increase in production over recent years. In 2011, the total output of the species exceeded 137,754 tons in China with 5.75% increase compared to that in 2010 (China Fishery Statistical Yearbook, 2012). However, sea cucumber farming is sustaining a frequent occurrence disease problem in practice, resulting in severe economic losses of the species aquaculture.[1]

Skin ulceration syndrome (SUS) is one of the most characteristic diseases in sea cucumber aquaculture because of its highly infectious and lethal rates.[2] The disease outbreak usually occurs in February to April with obvious syndrome of anorexia and shaking head at the first stage, followed ultimately by skin ulceration. In order to establish an effective disease control strategy, different pathogens like *Vibrio*, *Pseudomonas* and even virus have been characterized from a variety of diseased samples,[2−5] in which *Vibrio splendidus* was widely accepted as one of the major pathogens by many researchers. Thus far, many studies have been performed to explore some key members of molecules related to this bacterial exposure process.[6−10] Meantime, different immune stimulants (probiotics or their products) were also developed to improve sea cucumber growth and disease-resistance abilities.[11−14] To better understand the responsive mechanism, two digital gene expression (DGE) libraries from healthy and SUS samples were deep sequenced and two differential expressed microRNAs and 4,858 unigenes were identified in our previous works,[1,15] providing new evidence on this host−pathogen interaction process at global overview. However, how these differentially expressed molecules perturbing sea cucumber metabolic pathways and finally modulating the production of various metabolites remain largely unknown.

NMR-based metabonomics is a robust means to provide basic information on all containing metabolites in free solution by a uniformly concentration-dependent manner without extensive sample preparation.[16] Until now, no attempt has been made to address the linkage between metabolite richness and host−pathogen interaction in *A. japonicus*. In *Mytilus galloprovincialis*, *M. luteus* and *V. anguillarum* both induced disorders in osmotic regulation and energy metabolism in gill and hepatopancreas.[17,18] Liu et al. reported that *V. anguillarum* and *V. splendidus* induced imbalance of three physiological processes in clam *Ruditapes philippinarum* hepatopancreas, such as oxidative and immune stresses, energy metabolism and osmotic regulation.[19] In this work, a comparative study of the responsive profiles in muscle tissues from SUS-diseased and pathogen-challenged *A. japonicus* was investigated by NMR-based metabonomics to enhance our understanding of biological responses to pathogen infection and disease outbreak.

MATERIALS AND METHODS

Animals and Challenge Experiment. Twenty adult sea cucumbers *A. japonicus* (8−10 cm in length with average weight 100

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g) with the representative syndrome of skin ulceration were collected from indoor farms of Jinzhou Hatchery in May 2013. Identical numbers of sample without any diseased syndrome were also sampled to serve as control group. The environmental seawater met the quality criterion of class I water with temperature of 15−17 °C and salinity of 25−27 psu during these samples collected.

For *V. splendidus* challenge experiment, an overnight culture of bacteria was grown in 50 mL of liquid 2216E broth (pH 7.6) at 28 °C with shaking at 220 rpm. The bacteria were harvested by centrifuging at 3,000 rpm for 5 min, and then were resuspended in the same volume of filtered seawater for infection experiment. One hundred healthy adult sea cucumbers *A. japonicus* (similar size to natural diseased samples) were obtained from Bowang Aquaculture Company (Ningbo, China) and acclimatized in five plastic tanks (80 cm × 45 cm × 50 cm) with aerated seawater (salinity 30 psu) for 5 days. One tank served as control, and the other four tanks were supplied with overnight cultured *V. splendidus* to the final concentration of 10^7 CFU mL^{-1}. Muscles were dissected from individuals in each tank at 0, 24, 48, 72, and 96 h and quickly frozen by liquid nitrogen before metabolite extraction. Ten replicates were performed for each experimental sample from different time points as well as unchallenged ones.

Metabolite Extraction. Polar metabolites in *A. japonicus* (n = 10 for each treatment) muscle were extracted according to the protocol described by Lin et al. and Wu et al.[20,21] Briefly, each sample of muscle tissue (approximately 100 mg wet weight) was ground into tiny particles in liquid nitrogen, then homogenized into 1.125 mL extraction solution (0.44 mL of methanol, 0.525 mL of water and 0.2 mL of chloroform). Subsequently, the mixture was swirled and stilled for 10 min before centrifuging at 3000g for 5 min at 4 °C. The supernatant with polar metabolites was transferred and dried in a centrifugal concentrator. The samples were then redissolved in 600 μL of sodium phosphate buffer in D_2O following centrifugation (3000g, 5 min, 4 °C), a total of 550 μL of supernatant was transferred into 5 mm NMR tubes for NMR analysis.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra of metabolites from *A. japonicus* muscle were collected on a Bruker AV 500 NMR spectrometer recording at 500.18 MHz held at 25 °C as described previously.[22,23] Briefly, one-dimensional (1-D) ^{1}H NMR spectra were obtained using a standard 1D NOESY pulse sequence according to manufactory instructions. TopSpin 2.1 (Bruker) was manually employed to phase correct baseline and calibrate chemical shift of all ^{1}H NMR spectra at 0.0 ppm.

Preprocessing of ^{1}H NMR Data and Statistical Analysis. ^{1}H NMR spectra from SUS and *V. splendidus*-challenged samples were processed, put into corresponding software and further analyzed according to the method in the references.[24−29] One-way analysis of variance (ANOVA) was conducted on the *A. japonicus* between control and *V. splendidus*-challenged to test the statistical significance (P < 0.05) of separations.

RESULTS

Metabolomic Responses in Muscle Tissues of *A. japonicus* Challenged by *V. splendidus*. A typical ^{1}H NMR spectrum of the sea cucumber muscle metabolites after *V. splendidus* challenge for 24 h is shown in Figure 1. Different kinds of metabolites were detected in muscle tissues of *A. japonicus*, including a wide range of amino acids (alanine, glutamate, glutamine, glycine, isoleucine, leucine, phenylalanine, threonine and valine), two energy metabolism-related metabolites (ATP and glucose), two osmolytes (betaine and taurine), and two Krebs cycle-related metabolites (fumarate and succinate). All the ^{1}H NMR spectra were dominated by betaine (3.27 and 3.90 ppm), which is an important organic osmolyte in marine invertebrates (Table 1).

^{1}H NMR spectral data sets were further analyzed by PCA method, and significant difference was detected between control and *V. splendidus*-treated groups at 48 (P < 0.05), 72 (P < 0.05) and 96 h (P < 0.05), respectively (Figure 2). Furthermore, O-PLS-DA was also conducted on the ^{1}H NMR

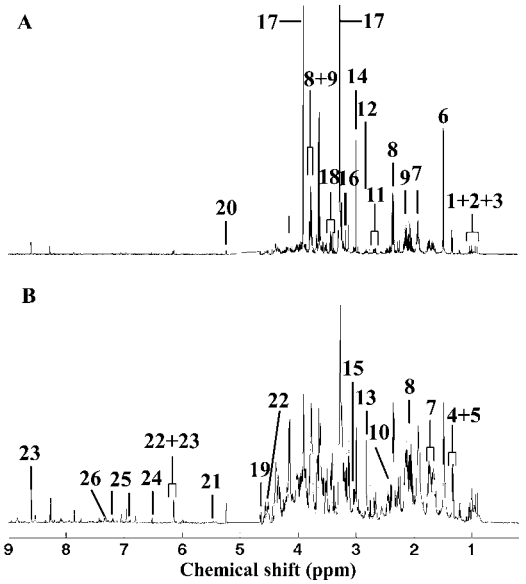

Figure 1. Representative 1-D ^{1}H NMR spectrum of unchallenged *Apostichopus japonicus* muscle in original (A) and glog transformed (B) forms. Key: (1) leucine, (2) isoleucine, (3) valine, (4) lactate, (5) threonine, (6) alanine, (7) arginine, (8) glutamate, (9) glutamine, (10) succinate, (11) malate, (12) dimethylamine, (13) dimethylglycine, (14) unknown (2.97 ppm), (15) lysine, (16) phosphocholine, (17) betaine, (18) taurine, (19) α-glucose, (20) β-glucose, (21) glycogen, (22) AMP, (23) ATP, (24) fumarate, (25) tyrosine and (26) phenylalanine.

Table 1. Metabolite Assignments of *Apostichopus japonicus* Muscle Revealed by the 1-D 500 MHz ^{1}H NMR Spectra

metabolite	chemical shift (ppm, multiplicity)[a]
valine	1.05 (d)
isoleucine	1.00 (d)
leucine	0.94 (t)
threonine	1.33 (d)
lactate	1.34 (d)
alanine	1.48 (d)
arginine	1.70 (m)
glutamate	2.05 (m)
glutamine	2.14 (m)
succinate	2.41 (s)
malate	2.67 (dd)
dimethylamine	2.75 (s)
dimethylglycine	2.91 (s)
lysine	3.03 (t)
phosphocholine	3.22 (s)
betaine	3.27 (s), 3.90 (s)
taurine	3.27 (t)
glucose	4.64 (d), 5.24 (d)
glycogen	5.40 (s)
AMP	4.01 (m), 6.13 (d)
ATP	4.25 (m), 6.14 (d)
fumarate	6.52 (s)
tyrosine	6.91 (d)
phenylalanine	7.34 (m)

[a]s = singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet.

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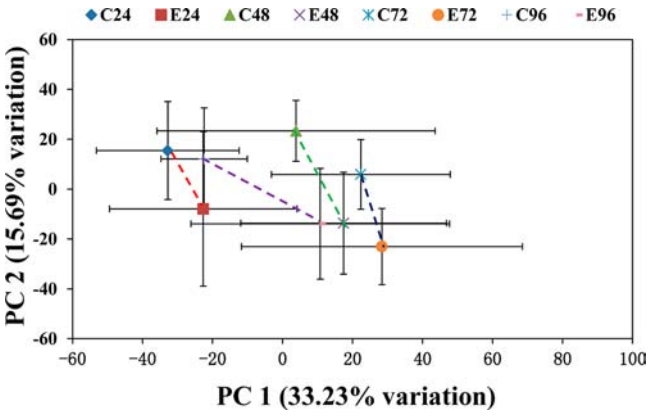

Figure 2. PCA plots of ^{1}H NMR spectra from control and *V. splendidus*-challenged groups sampled at 24, 48, 72, and 96 h in *A. japonicus* muscles. All data were expressed as means ± SD (n = 10) for each group of *A. japonicus* samples.

spectral data sets from control and *V. splendidus*-challenged samples at each sampling time point for metabolic biomarker discovery related to *V. splendidus* challenge (Figure 3). The scores plots (Figure 3A,C,E) derived from O-PLS-DA indicated clear classifications between control and *V. splendidus*-challenged groups after *V. splendidus* infection for 48, 72, and 96 h, respectively, with reliable Q^2 values (>0.4). However, both unsupervised (PCA) and supervised (O-PLS-DA) pattern recognition methods did not result in significant separation between control and *V. splendidus*-challenged groups at the first sampling time point, 24 h. It suggested that *V. splendidus* did not induce obvious biological effects in *A. japonicus* muscle after infection for 24 h.

Based on the loading plot of O-PLS-DA (Figure 3B), the concentrations of AMP, glucose and three amino acids (isoleucine, leucine and valine) were significantly increased in *V. splendidus*-challenged *A. japonicus* muscle samples after infection for 48 h (P < 0.05), while ATP was obviously decreased. In comparison with the metabolic profile at 48 h, a completely different metabolic response was detected in *A.*

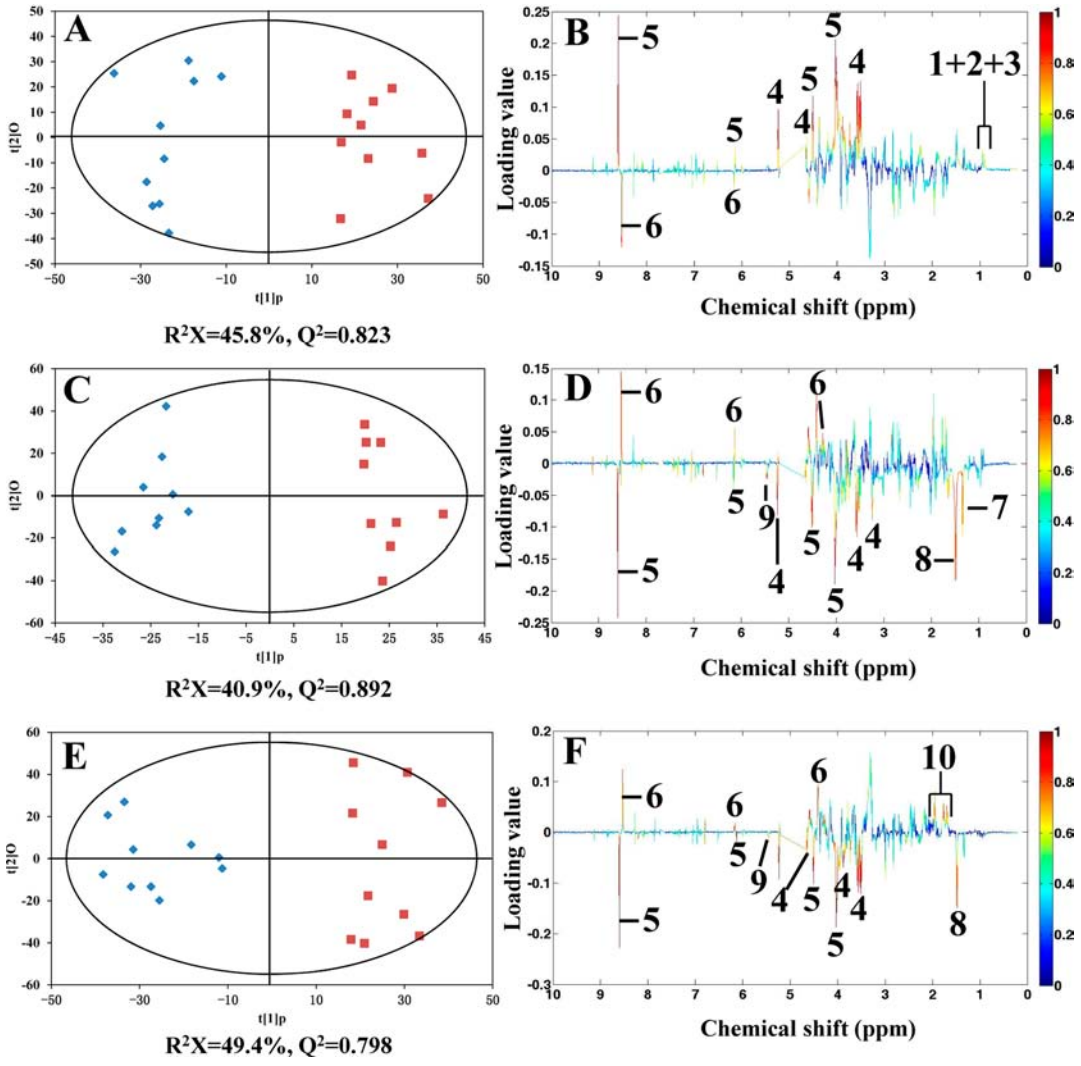

Figure 3. OPLS-DA plots of ^{1}H NMR spectra from control (blue ◆) and *V. splendidus*-challenged *A. japonicus* groups (red ■) muscles at 48 h (A), 72 h (C) and 96 (E) and corresponding coefficient plots (B), (D) and (F). The significantly variations of metabolite between control and experimental groups were indicated with color. Abundant metabolites in experimental samples were shown by peak in the positive direction, and that in control group are presented as peaks in the negative direction. Key: (1) leucine, (2) isoleucine, (3) valine, (2) threonine, (3) arginine, (4) glucose, (5) AMP, (6) ATP, (7) threonine, (8) alanine, (9) glycogen and (10) arginine.

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japonicus muscle tissues at 72 h. As shown in the loading plot of O-PLS-DA (Figure 3D), two amino acids, alanine and threonine, were apparently decreased together with glycogen. Interestingly, other metabolites including ATP, AMP and glucose were contrarily altered in *V. splendidus*-challenged *A. japonicus* muscle tissue samples at 72 h compared with those at 48 h. After challenge with *V. splendidus* for 96 h, the metabolic profiles presented some similar metabolic biomarkers including elevated ATP and depleted alanine, AMP, glucose and glycogen (Figure 3F). The level of threonine recovered to the control. However, arginine was uniquely increased in *V. splendidus*-challenged *A. japonicus* samples after infection for 96 h.

Metabolomic Responses in Muscle Tissues of SUS-Diseased *A. japonicus* Samples. ^{1}H NMR spectral data sets were also analyzed by O-PLS-DA from healthy and SUS-diseased groups sampled from indoor ponds of Jinzhou Hatchery in May 2013 (Figure 4). The scores plot (Figure 4A) displayed a reliable classification between healthy and SUS-diseased groups, with a high Q^2 value (>0.9). From the observation of the loading plot (Figure 4B), the SUS-diseased group presented significant metabolic differences compared with the metabolic profiles from healthy *A. japonicus* samples, including relatively high levels of glucose and glycogen and low levels of threonine, alanine, arginine, glutamate, glutamine, taurine and ATP.

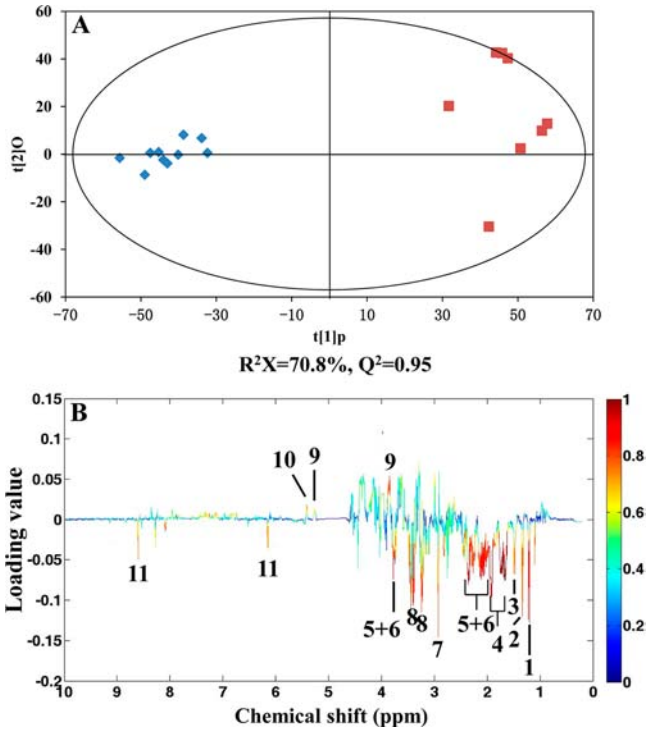

Figure 4. O-PLS-DA plots (A) of ^{1}H NMR spectra from healthy (blue ◆) and SUS-diseased (red ■) field samples, and corresponding coefficient plot (B). The significant variations of metabolite between healthy and SUS groups are indicated with color. Abundant metabolites in SUS-diseased samples are shown by peak in the positive direction, and those in healthy group are presented as peaks in the negative direction. Key: (1) unknown 1 (1.18 ppm), (2) threonine, (3) alanine, (4) arginine, (5) glutamate, (6) glutamine, (7) unknown 2 (2.97 ppm), (8) taurine, (9) glucose, (10) glycogen and (11) ATP.

DISCUSSION

As shown in Figure 1, betaine is the most abundant metabolite in *A. japonicus* muscle tissues. Betaine is a known organic osmolyte that is synthesized in a two-step reaction from choline: choline → betaine aldehyde + NAD^+ → betaine + NADH in marine invertebrates to regulate osmotic balance between intracellular and external saline environments.[29] In the reaction of converting choline to betaine, the initial step is usually catalyzed by choline dehydrogenase, subsequently by betaine aldehyde dehydrogenasethe. However, other osmolytes including glycine and taurine were found at relatively low concentrations in *A. japonicus* samples (Figure 1). This implied that betaine was the main organic osmolyte playing an important role in osmotic regulation rather than other osmolytes (e.g., taurine, glycine) and therefore was observed in *A. japonicus* muscle tissues at high concentrations.[29]

After challenge with *V. splendidus* for 48 h, three branched chain amino acids of isoleucine, leucine and valine were significantly elevated in *A. japonicus* muscle tissues. Ji et al. reported that *V. anguillarum* challenge could induce a similar profile of branched chain amino acids in gills of mussel *Mytilus galloprovincialis*.[17] In marine invertebrates, amino acids are considered to be major players in regulating energy metabolism and osmotic pressure.[30] Usually, the high concentrations of amino acids can be used as osmolytes to regulate the intracellular osmolarity with the environment.[30] In addition, energy production is an everlasting event when organisms are exposed to various stresses, which is usually produced by oxidation of amino acids and glucose. This catabolic period is usually accompanied by the degradation of glycogen and protein. Since no osmolytes were significantly altered in *V. splendidus*-challenged *A. japonicus* samples at 48 h, the elevated amino acids might be related to a reduced energy demand together with the elevated glucose (enhanced gluconeogenesis) and depleted ATP. Obviously, the increased AMP resulted in the enhanced hydrolysis of ATP. Specifically, all branched chain amino acids have availability on the immune system to function by incorporating into proteins.[31] Upon pathogenic infection, there might be a remarkable increase in demand for these branched chain amino acids as substrates in order to maintain the activating of the host immune system.[31] These amino acids then provide needed energy via the pathways of amino acid oxidation and are also used as the precursors for the biosynthesis of new protective molecules.[31] In our case, therefore, the branched chain amino acids were increased to deal with the infection of *V. splendidus* in *A. japonicus* muscle tissues after challenge for 48 h.

For the *A. japonicus* samples challenged by *V. splendidus* at 72 h, the mode of action induced by *V. splendidus* was completely different because of the contrarily altered metabolic biomarkers compared with those in *V. splendidus*-challenged *A. japonicus* samples at 48 h. The decreased amino acids (alanine and threonine), glucose and glycogen and increased ATP meant the promoted oxidation of these metabolites for the enhanced energy demand. Previous studies have found that a significant ($P < 0.05$) increase in the energy consumption was common in hepatopancreas, gill, mantle and muscle tissues from *Vibrio anguillarum*-challenged scallop *Chlamys farreri* under high temperature and ammonia-N exposures for 96 h.[32,33] Evidence suggests that organisms need more energy to deal with exogenous stressors, such as pathogens.[32,33] This is totally agreement with our present results, which show a significant

increase in energy demand in *V. splendidus*-challenged *A. japonicus* samples at 72 h, as indicated by decreased glucose, glycogen and amino acids and increased ATP. The metabolic responses of *A. japonicus* samples at 96 h were similar to those at 72 h, except decreased threonine and increased arginine, which suggested that *V. splendidus* infection caused a significant increase in energy demand in *A. japonicus* samples at 96 h. Both ATP and arginine are involved in the reaction, ATP + arginine ↔ ADP + phosphoarginine, which is catalyzed by arginine kinase (EC 2.7.3.3). Therefore, the uniquely altered metabolite, arginine, was increased consistently with ATP.

We originally expected to discover potential metabolic biomarkers indicating the disease caused by *V. splendidus*. Unfortunately, the metabolic differences between SUS-diseased and healthy *A. japonicus* samples with the metabolic biomarkers derived from the lab experiment. Basically, the metabolic differences clearly indicated reduced energy demand (glucose, glycogen, ATP and amino acids) and disturbance in osmotic regulation (taurine) in the SUS-diseased group. Obviously, these metabolic differences were not consistent with those from the lab experiment. This finding was supported by the fact that obvious syndromes of natural SUS-diseased samples like skin ulceration were not detected in *V. splendidus*-challenged sea cucumber during the whole laboratory challenge experiment. Meanwhile, the different living environment for the two examined samples was also accounted for in this result. As it is known, the conditions in laboratory experiment were identical for both control and pathogen-challenged animals. For the field samples, however, the environmental factors (e.g., salinity, temperature, strains of pathogens, food, light, exposure time) could not be controlled strictly. More importantly, mixed infection by different pathogens was also not excluded from the possibilities for these divergent metabolic responses. Other than *V. splendidus*, *Pseudomonas* and even spherical virus had been demonstrated to be potential pathogens for SUS-diseased sea cucumber.[1] Deng et al. had also separated six predominant isolates of bacteria from the SUS-diseased sea cucumbers. All of these bacteria could successfully infect the healthy samples with the typical morphological syndrome of natural SUS individuals.[2] Therefore, the natural SUS-diseased sea cucumbers might be suffered from a variety of pathogens' combined action which led to metabolic biomarkers different from *V. splendidus*-challenged. To better address this speculation, metabolic responses toward individual and combined pathogen exposures would be investigated in our further work.

In summary, the major change metabolites in *V. splendidus*-challenged sea cucumbers were betaine, branched chain amino acids (alanine, threonine, argimine), glucose, glycogen, ATP, while the metabolite pattern of SUS disease is composed of glucose and glycogen in abundance and branched chain amino acids (alanine, threonine, argimine, glutamate, glutamine), taurine and ATP in low levels. The divergent metabolic responses from SUS-diseased and *V. splendidus*-challenged sea cucumbers revealed the complicated process between disease outbreaks and pathogen infection under the varied marine environments. The metabolic biomarkers indicating *V. splendidus* challenge were not usable for the prediction of SUS disease in practice. In order to better understand their connection, multiple-omics techniques would be applied to our further work to deal with the crosstalk between pathogen infection and disease outbreaks.

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Notes

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REFERENCES

(1) Li, C.; Feng, W.; Qiu, L.; Xia, C.; Su, X.; Jin, C.; Zou, T.; Zeng, Y.; Li, T. Characterization of skin ulceration syndrome associated microRNAs in sea cucumber *Apostichopus japonicus* by deep sequencing. *Fish Shellfish Immunol.* **2012**, *33*, 436−441.

(2) Deng, H.; He, C.; Zhou, Z.; Liu, C.; Tan, K.; Wang, N.; Jiang, B.; Gao, X.; Liu, W. Isolation and pathogenicity of pathogens from skin ulceration disease and viscera ejection syndrome of the sea cucumber *Apostichopus japonicus*. *Aquaculture* **2009**, *287*, 18−27.

(3) Zhang, C.; Wang, Y.; Rong, X. Isolation and identification of causative pathogen for skin ulcerative syndrome in *Apostichopus japonicus*. *J. Fish. China* **2006**, *30*, 118−123 (in Chinese).

(4) Wang, P.; Chang, Y.; Yu, J.; Li, C.; Xu, G. Acute peristome edema disease in juvenile and adult sea cucumbers *Apostichopus japonicus* (Selenka) reared in North China. *J. Invertebr. Pathol.* **2007**, *96*, 11−17.

(5) Liu, H.; Zheng, F.; Sun, X.; Hong, X.; Dong, S.; Wang, B.; Tang, X.; Wang, Y. Identification of the pathogens associated with skin ulceration and peristome tumescence in cultured sea cucumbers *Apostichopus japonicus* (Selenka). *J. Invertebr. Pathol.* **2010**, *105*, 236−242.

(6) Cong, L.; Yang, X.; Wang, X.; Tada, M.; Lu, M.; Liu, H.; Zhu, B. Characterization of an i-type lysozyme gene from the sea cucumber *Stichopus japonicus*, and enzymatic and nonenzymatic antimicrobial activities of its recombinant protein. *J. Biosci. Bioeng.* **2009**, *107*, 583−588.

(7) Zhou, Z.; Sun, D.; Yang, A.; Dong, Y.; Chen, Z.; Wang, X.; Guan, X.; Jiang, B.; Wang, B. Molecular characterization and expression analysis of a complement component 3 in the sea cucumber (*Apostichopus japonicus*). *Fish Shellfish Immunol.* **2011**, *31*, 540−547.

(8) Sun, H.; Zhou, Z.; Dong, Y.; Yang, A.; Jiang, B.; Gao, S.; Chen, Z.; Guan, X.; Wang, B.; Wang, X. Identification and expression analysis of two Toll-like receptor genes from sea cucumber (*Apostichopus japonicus*). *Fish Shellfish Immunol.* **2013**, *34*, 147−158.

(9) Lu, Y.; Li, C.; Zhang, P.; Shao, Y.; Su, X.; Li, Y.; Li, T. Two adaptor molecules of MyD88 and TRAF6 in *Apostichopus japonicus* Toll signaling cascade: molecular cloning and expression analysis. *Dev. Comp. Immunol.* **2013**, *41*, 498−504.

(10) Han, L.; Yuan, Z.; Dahms, H.; Li, Q.; Zhang, Q.; Wu, R.; Tan, J.; Zou, X.; Hou, L. Molecular cloning, characterization and expression analysis of a C-type lectin (AJCTL) from the sea cucumber *Apostichopus japonicus*. *Immunol. Lett.* **2012**, *143*, 137−145.

(11) Sun, Y.; Jin, L.; Wang, T.; Xue, J.; Liu, G.; Li, X.; You, J.; Li, S.; Xu, Y. Polysaccharides from *Astragalus membranaceus* promote phagocytosis and superoxide anion (O2−) production by coelomocytes from sea cucumber *Apostichopus japonicus in vitro*. *Comp. Biochem. Physiol., Part C* **2008**, *147*, 293−298.

(12) Zhang, Q.; Ma, H.; Mai, K.; Zhang, W.; Liufu, Z.; Xu, W. Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the

growth performance, non-specific immunity of sea cucumber, *Apostichopus japonicus*. *Fish Shellfish Immunol.* **2010**, *29*, 204−211.
(13) Ma, Y.; Liu, Z.; Yang, Z.; Li, M.; Liu, J.; Song, J. Effects of dietary live yeast *Hanseniaspora opuntiae* C21 on the immune and disease resistance against *Vibrio splendidus* infection in juvenile sea cucumber *Apostichopus japonicus*. *Fish Shellfish Immunol.* **2013**, *34*, 66−73.
(14) Gu, M.; Ma, H.; Mai, K.; Zhang, W.; Bai, N.; Wang, X. Effects of dietary β-glucan, mannan oligosaccharide and their combinations on growth performance, immunity and resistance against *Vibrio splendidus* of sea cucumber, *Apostichopus japonicus*. *Fish Shellfish Immunol.* **2011**, *31*, 303−309.
(15) Zhang, P.; Li, C.; Zhu, L.; Su, X.; Li, Y.; Jin, C.; Li, T. *De novo* assembly of the sea cucumber *Apostichopus japonicus* hemocytes transcriptome to identify miRNA targets associated with skin ulceration syndrome. *PLoS One* **2013**, DOI: 10.1371/journal.-pone.0073506.
(16) Solanky, K. S.; Burton, I. W.; MacKinnon, S. L.; Walter, J. A.; Dacanay, A. Metabolic changes in Atlantic salmon exposed to *Aeromonas salmonicida* detected by ^{1}H-nuclear magnetic resonance spectroscopy of plasma. *Dis. Aquat. Org.* **2005**, *65*, 107−114.
(17) Ji, C.; Wu, H.; Wei, L.; Zhao, J.; Wang, Q.; Lu, H. Responses of *Mytilus galloprovincialis* to bacterial challenges by metabolomics and proteomics. *Fish Shellfish Immunol.* **2013**, *35*, 489−498.
(18) Wu, H.; Ji, C.; Wei, L.; Zhao, J.; Lu, H. Proteomic and metabolomic responses in hepatopancreas of *Mytilus galloprovincialis* challenged by *Micrococcus luteus* and *Vibrio anguillarum*. *J. Proteomics* **2013**, *94*, 54−67.
(19) Liu, X.; Ji, C.; Zhao, J.; Wu, H. Differential metabolic responses of clam *Ruditapes philippinarum* to *Vibrio anguillarum* and *Vibrio splendidus* challenges. *Fish Shellfish Immunol.* **2013**, DOI: 10.1016/j.fsi.2013.09.014.
(20) Lin, C.; Wu, H.; Tjeerdema, R. S.; Viant, M. R. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics* **2007**, *3*, 55−67.
(21) Wu, H.; Southam, A. D.; Hines, A.; Viant, M. R. High throughput tissue extraction protocol for NMR and mass spectrometry based metabolomics. *Anal. Biochem.* **2008**, *372*, 204−212.
(22) Liu, X.; Zhang, L.; You, L.; Yu, J.; Zhao, J.; Li, L.; Wang, Q.; Li, F.; Li, C.; Liu, D.; Wu, H. Differential toxicological effects induced by mercury in gills from three pedigrees of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. *Ecotoxicology* **2011**, *20*, 177−186.
(23) Liu, X.; Zhang, L.; You, L.; Cong, M.; Zhao, J.; Wu, H.; Li, C.; Liu, D.; Yu, J. Toxicological responses to acute mercury exposure for three species of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. *Environ. Toxicol. Pharmacol.* **2011**, *31*, 323−332.
(24) Parsons, H. M.; Ludwig, C.; Gunther, U. L.; Viant, M. Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. *BMC Bioinformatics* **2007**, *8*, 234.
(25) Xu, L. *Methods of chemometrics*; Science Press: Beijing, 2004; pp 221−227.
(26) Feng, J.; Li, J.; Wu, H.; Chen, Z. Metabolic responses of HeLa cells to silica nanoparticles by NMR-based metabolomic analyses. *Metabolomics* **2013**, DOI: 10.1007/s11306-013-0499-8.
(27) Feng, J.; Zhao, J.; Hao, F.; Chen, C.; Bhakoo, K.; Tang, H. NMR-based metabonomics analyses of the effects of ultrasmall superparamagnetic particles of iron oxide (USPIO) on macrophage metabolism. *J. Nanopart. Res.* **2011**, *13*, 2049−2062.
(28) Fan, W. M. T. Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *28*, 161−219.
(29) Ferrino, L. A.; Pierce, S. K. Betaine aldehyde dehydrogenase kinetics partially account for oyster population differences in glycine betaine synthesis. *J. Exp. Zool.* **2000**, *286*, 238−249.
(30) Viant, M. R.; Rosenblum, E. S.; Tjeerdema, R. S. NMR-based metabolomics: A powerful approach for characterizing the effects of environmental stressors on organism health. *Environ. Sci. Technol.* **2003**, *37*, 4982−4989.
(31) Calder, P. C. Branched chain amino acids and immunity. *J. Nutr.* **2006**, *136*, 288S−293S.
(32) Wang, X.; Wang, L.; Zhang, H.; Ji, Q.; Song, L.; Qiu, L.; Zhou, Z.; Wang, M.; Wang, L. Immune response and energy metabolism of *Chlamys farreri* under *Vibrio anguillarum* challenge and high temperature exposure. *Fish Shellfish Immunol.* **2012**, *33*, 1016−1026.
(33) Wang, X.; Wang, L.; Yao, C.; Qiu, L.; Zhang, H.; Zhi, Z.; Song, L. Alternation of immune parameters and cellular energy allocation of *Chlamys farreri* under ammonia-N exposure and *Vibrio anguillarum* challenge. *Fish Shellfish Immunol.* **2012**, *32*, 741−749.