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RESEARCH PAPER

Gut Microbiota and Metabolic Phenotype of *Portunus Trituberculatus*

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Abstract: 16S rRNA gene high-throughput sequencing and nuclear magnetic resonance (NMR)-based metabolomics techniques were established for the composition analysis of gut microbiota and metabolites of *Portunus trituberculatus*. Swimming crab gut samples were used for the extraction of bacterial genomic DNA, followed by amplification of hypervariable domain V3-V4 of 16 S rRNA gene. The obtained PCR products were analyzed on an Illumina MiSeq platform. The bacterial phonotypes were then clustered and assigned. The results showed that swimming crab gut sample was dominated by Proteobacteria, Bacteroidetes, Fusobacteria, Tenericutes, and Acidobacteria at the phylum level, among which, *Photobacterium*, *Paludibacter*, and *Propionigenium* had the maximum abundance at the genus level. Crab intestinal samples were extracted with methanol/H_2O (2:1, *V/V*) solution under shaking with a TissueLyser. After the removal of methanol, the resultant supernatants were lyophilized. Each of lyophilized extract was dissolved into Na^+/K^+ phosphate buffer and then centrifuged at high speed for NMR analysis. A standard Noesypr1D was used to acquire ^1H NMR spectra. The 90° pulse length was adjusted to approximately 10 μs. The recycle delay and mixing time were set to 2 s and 100 ms in sequence. The spectral width was set to 20 ppm. Sixty-four transients were collected into 32768 data points for each spectrum. A range of two-dimensional NMR spectra were acquired for the resonance assignment. The results showed that swimming crab gut metabolome comprised 30 metabolites including some amino acids, organic acids and amines. In summary, this study provided a method for the systematic analyses of the compositions of gut bacterial community and metabolites of swimming crab.

Key Words: *Portunus trituberculatus*; Gut microbiota; High-throughput sequencing; Metabolic phenotype; Nuclear magnetic resonance

1 Introduction

Intestinal microbial communities are intricately linked to host's health[1]. They play a central role in nutrient and energy cycling of host[2]. The previous investigations of crustacean indicated that gut microbiota were responsible in part for organ development[3], nutrition[4], immunity[5], and diseases[6]. For example, characteristic gut bacteria in mud crab *Scylla paramamosain* was closely associated with its health[7]. Given the importance of gut bacteria, three *Bacillus* strains with antagonistic activity against *Vibrio parahaemolyticus* were supplemented into juvenile mud crab diets, resulting in the enhanced immune response and protection of crab against vibrio[5]. For another example, the habitat, health, and diets of Chinese mitten crabs (*Eriocheir sinensis*) play an importance role in shaping the symbiotic gut bacteria pattern[8]. The swimming crab *Portunus trituberculatus* is one of the most important economic animals for marine aquaculture in China. It widely spreads in the marginal seas including the Bohai Sea, Yellow Sea, East China Sea and South China Sea[9]. However, basic information on intestinal microbiota of swimming crab is still lacking.

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The method of microbial ecological research has been developed with a shift from culture-dependence to non-culture-dependence. Nowadays, the methods for analysis of crab gut bacterial community include 16S rRNA gene clone library and metagenomics sequencing. For instance, a clone library analysis was previously employed for characterizing intestinal bacterial community of Chinese mitten crab (*Eriocheir sinensis*) farmed in Chongming Island. Crab gut contained 90%–95% of the phylotypes affiliated with Bacteroidetes and Proteobacteria[8]. In contrast, a metagenomics sequencing of 16S rRNA gene showed a different gut bacterial composition of Chinese mitten crabs, which dominated by Tenericutes, Bacteroidetes, Proteobacteria, and Firmicutes[10,11]. The same method was applied to analyze the effects of pectin and xylan on the intestinal microflora structure of Chinese mitten crabs[12]. However, the results showed that the abundant bacteria were Proteobacteria, Firmicutes, Bacteroidetes, and Fusobacteria, which were different from those of Chen[10] and Zhang[11]. The reason was likely due to the different cultural environment and diet. Moreover, the denaturing gradient gel electrophoresis and clone library analysis techniques were jointly used to analyze the intestinal microbial community of mud crab Scylla paramamosain[7]. The results showed that the abundance of *Bacteriodes* in wild and pond-raised healthy crabs was much higher than that in diseased crabs. Cyanobacteria inhabited unique in diseased crabs, whereas Firmicutes was only found in healthy crabs.

In general, changes in symbiotic intestinal microbial communities may cause an altered metabolic phenotype (metabotype) of the host. This is because the microbiota not only provide complementary metabolic pathways, but also produce secondary metabolites that can activate enzyme systems of host[13]. However, most studies of intestinal microbial metabolism focused on mammals, such as humans[14]. In contrast, less attention was paid to crabs, despite the fact that crabs were a major agricultural product with high economic value worldwide. The knowledge of intestinal microbial community and metabolic phenotype is a prerequisite for the understanding of complex interaction between crab and its symbiotic gut microbiome.

A nuclear magnetic resonance (NMR)-based metabolomic technique was widely applied in the human intestinal metabolic phenotype[14] and microbial metabolome[15]. Therefore, a metagenomics sequencing of 16S rRNA gene coupled with NMR-based metabolomic technique could be used for comprehensive analysis of the intestinal bacterial community and metabolic phenotype. However, little has been reported on the metabolomic and bacterial analysis of swimming crab gut using these two methods. So far, there is a lack of information of the crab gut microbiota and metabolome composition.

In this study, we systematically analyzed the bacterial community structure and assigned the metabolite spectral features of swimming crab gut with 16S rRNA gene amplicon pyrosequencing and NMR-based metabolomic technique. The aim of this work is to provide the basic information on the structure of the intestinal bacterial community and crustacean-microbial cometabolites.

2 Experimental

2.1 Instruments and reagents

The NMR measurements were performed on a Bruker Avance III 600 MHz spectrometer equipped with an inverse detection cryogenic probe (Bruker Biospin, Rheinstetten, Germany). Pyrosequencing was conducted on an Illumina MiSeq platform (Illumina, San Diego, USA). The gut cells were broken using a TissueLyser (Qiagen/Retsch, Germany). PowerFecal™ DNA Isolation kit was purchased from MO BIO Laboratories (Carlsbad, CA, USA). A PCR fragment purification kit was purchased from TaKaRa Biotechnology (Dalian) Co. Ltd. (Dalian, China). Methanol, $NaH_2PO_4{\cdot}2H_2O$ and $K_2HPO_4{\cdot}3H_2O$ were purchased from Guoyao Chemical Co. Ltd. (Shanghai, China) all in analytical grade. Sodium 3-trimethylsilyl [2,2,3,3-$^2D_4$] propionate (TSP) and deuterated water (D_2O, 99.9% D) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.2 Preparation of extraction solvent and phosphate buffer

Metabolite extraction solvent was an aqueous methanol (methanol-ddH_2O, 2:1, *V*/*V*). Phosphate buffer (K_2HPO_4/NaH_2PO_4, 0.1 M, pH 7.4), containing 10% D_2O (*V*/*V*) and 0.005% TSP (*w*/*V*), was prepared in ddH_2O[16].

2.3 Sample preparations and extraction

Three live swimming crabs (about 200 g) were purchased from a local supermarket. The crabs were kept on the ice and brought into the laboratory in 1 h. The crabs were washed thrice with sterile water to minimize the likelihood of detecting surface-associated microbes before intestine isolation. Three intestinal samples were collected and ground with liquid nitrogen. Each sample was divided into two portions. One portion (about 100 mg) was used for bacterial composition analysis, and the another portion (about 300 mg) for NMR analysis. All samples were kept at –80 ºC until further analysis.

2.4 DNA extraction, bacterial 16S rRNA gene amplification and MiSeq sequencing

Total genomic DNA was extracted using the PowerFecal™ DNA Isolation kit for the intestine according to the manufacturer's protocols. The quality and quantity of the total