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Metabolism of branched-chain amino acids revealed by transcriptome analysis in *Vibrio alginolyticus*

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ABSTRACT

Branched-chain amino acids (BCAAs) play important roles in nitrogen metabolism. However, little is known about the metabolism of BCAAs in the fish pathogen *Vibrio alginolyticus*. In this study, the global gene expression patterns of *V. alginolyticus* ZJ-T cultured in M63 minimal medium supplemented with ammonium sulfate or with three BCAAs (isoleucine, leucine and valine) as nitrogen source were evaluated by transcriptome analysis. The results revealed that 311 genes are up-regulated ($|\log_2(\text{Fold Change})| > 1$), which are involved in the pathways of flagellar assembly, bacterial chemotaxis and oxidative phosphorylation *etc*, and meanwhile 251 genes are down-regulated, which are involved in the pathways of BCAAs biosynthesis, selenocompound metabolism and C5-branced dibasic acid metabolism *etc*. This study contributes to the understanding of the BCAAs metabolism in the *Vibrios*.

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1. Introduction

Branched-chain amino acids (BCAAs: Isoleucine, leucine and valine (ILV) are essential elements in both prokaryotic and eukaryotic organisms. They play diverse roles in metabolism and physiology. It was reported that BCAAs are required for protein synthesis and turnover, signaling transduction and glucose metabolism, as well as fatty acid oxidation (Babchia et al., 2010; Monirujjaman and Ferdouse, 2014; Yoshiharu et al., 2015). BCAAs were also considered as important players in the immune system and in brain function (Brosnan and Brosnan, 2006; Burrage et al., 2014; Fernstrom, 2005; Mattick et al., 2013; Yamamoto et al., 2016). In bacteria, they were reported to be vital for both growth and virulence (Kaiser et al., 2016).

The BCAAs biosynthesis pathway is presented in microorganisms and in plants, but not in animals. Therefore, animals must acquire these amino acids from diets (Holecek, 2010; Yoshiharu et al., 2015; Zabalza et al., 2013), which makes the BCAAs synthesis pathway as an attractive target for antimicrobial drug development (Richie et al., 2013; Zabalza et al., 2013). Extensive studies on BCAAs metabolism for many bacteria were reported previously (Guo et al., 2015; Hou et al., 2012; Yamamoto et al., 2016). In Vibrio, the leucine-responsive regulatory protein (Lrp) was reported to be a global regulator protein and it regulates the biosynthesis of leucine as well as valine and isoleucine (Lintner et al., 2008). Additionally, acetone formation was found to be a new pathway for Leucine Catabolism in Vibrio family (Nemecek-Marshall et al., 1999). However, the mechanism of BCAAs metabolism is still largely unknown in Vibrio species.

Previously, a fish pathogen *Vibrio alginolyticus* ZJ-T was isolated and characterized. The whole genome sequence of this strain was determined (Yiqin et al., 2016). In this study, the transcriptomes of ZJ-T grown in M63 minimal medium with ammonium sulfate or with BCAAs as nitrogen source were analyzed comparatively. The result will contribute to a more comprehensive understanding of the metabolic mechanism of BCAAs in *V. alginolyticus* and may help for the development of drugs that targeting the BCAAs metabolism pathway.

2. Data description

2.1. Bacteria culture condition and RNA extraction

Three single colonies from *V. alginolyticus* ZJ-T were cultured in Luria-Bertani salt (LBS) rich medium for overnight. The cells were collected by centrifugation and were washed twice with M63 minimum medium without D-glucose and $(NH_4)_2SO_4$ (Unless otherwise indicated, M63 was made according to the following recipe: 3 $g \cdot L^{-1}$ KH_2PO_4, 7 $g \cdot L^{-1}$ K_2HPO_4, 0.5 × 10^{-3} $g \cdot L^{-1}$ $FeSO_4$, 30 $g \cdot L^{-1}$ NaCl, 2 × 10^{-3} M $MgSO_4$, 5 × 10^{-3} $g \cdot L^{-1}$ thiamine, 4 $g \cdot L^{-1}$ D-glucose, 2 $g \cdot L^{-1}$ $(NH_4)_2SO_4$). Then the cells were diluted to OD_{600} = 0.1 with M63 and

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M63 without $(NH_4)_2SO_4$ and BCAAs (20 mM isoleucine, 20 mM leucine and 20 mM valine) were added as nitrogen source. Cultures were then incubated at 30 °C with continuous shaking at 200 rpm for 5 h to reach the log phase. Equal amount of cells were harvested from each sample and total RNA was isolated using RNAiso Plus (Takara Bio Inc.) according to the manufacturer's instructions. The RNA degradation and contamination was monitored on 1% agarose gels and RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Then RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Three RNA samples from each medium were pooled for RNA-seq analysis.

2.2. Library preparation for strand-specific transcriptome sequencing

A total amount of 3 μg RNA per mixed sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, rRNA was removed using a specialized kit and mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. In the reaction buffer, dNTPs with dTTP were replaced by dUTP. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.3. Clustering and sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform and paired-end reads were generated. MIxS descriptors are presented in Table 1.

2.4. Identification of differentially expressed genes (DEGs)

Primary sequencing data produced by Illumina HiSeq 2500 were subjected to quality control. Filtered quality reads were mapped to the reference genome sequence of *V. alginolyticus* ZJ-T (Yiqin et al., 2016) using Bowtie2-2.2.3 (Langmead and Salzberg, 2012). HTSeq v0.6.1 was used to count the reads numbers that mapped to each gene. Then Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) value of each gene was calculated based on the length of the gene and reads count mapped to this gene (Trapnell et al., 2010). Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of the two conditions was performed using the DEGSeq R package (1.20.0). the *P*-values were adjusted using the Benjamini & Hochberg method. Corrected *P*-value of 0.005 and log2 (Fold change) of 1 were set as the threshold for significantly differential expression.

Table 1
MIxS descriptors.

Item	Description
Investigation_type	Prokaryote
Project_name	*Vibrio alginolyticus* ZJ-T transcriptome cultured with or without ILV
Collected_by	Chang Chen
Collection_date	2005
Sample source location	China (Zhanjiang, Guangdong)
Organism	*Vibrio alginolyticus* ZJ-T
Isolation environment	Sea coast
Biome	ENVO: 01000320
Feature	ENVO: 01000007
Material	ENVO: 00000303
Genome information	GenBank accession no. CP016224.1 and CP016224.2 (Yiqin et al., 2016)
Sequencing method	Illumina
Genome coverage	200×
Transcriptome platform	Illumina HiSeq2500
Sample material	*V. alginolyticus* ZJ-T pure culture in M63 minimal medium
Data format	Raw data: .fq.gz files
Experimental factors	Cultivation with or without ILV
Experimental features	Comparison transcriptome analysis to get a comprehensive understanding of ILV synthesis in *V. alginolyticus*

Overall, 562 genes were differentially expressed when the bacteria were cultured in M63 supplied with either BACCs or $(NH4)_2SO_4$ as nitrogen source, in which 311 genes were up-regulated and 251 were down-regulated (Figs. 1–2, Supplementary Table S1). KOBAS software was used to test the statistical enrichment of differentially expressed genes in KEGG pathways and 92 up-regulated genes were enriched into 48 pathways and 97 down-regulated genes were enriched into 60 pathways (Supplementary Table S2–S3). The first 20 pathways with most enriched up- and down-regulated genes were shown in Figs. 1 and 2, respectively.

The results showed that BCAAs affect many metabolism pathways, especially nitrogen and carbon metabolism (Figs. 1–2). Notably, addition of BCAAs in the culture medium generally promoted flagellar assembly, bacterial chemotaxis and oxidative phosphorylation (Fig. 1). It indicates that BCAAs may play critical roles in motility and energy production in *V. alginolyticus*, which is in accordance with the studies in other organisms. As in *Pseudomonas aeruginosa*, all of the three BCAAs promoted the biofilm formation and repressed the swarming motility to different levels (Bernier et al., 2011). In *Streptococcus mutans*, BCAAs were reported to enhance the ability of CodY to bind to the promoter region of the acetate kinase gene (*ackA*) that converts pyruvate into acetate with the concomitant generation of ATP (Kim and Burne, 2017). As shown in Fig. 2, our results suggested that carbon metabolism may be inactivated while free BCAAs were added to media to serve as the carbon source. On the other side, BCAAs turn down the pathways including BCAAs biosynthesis, selenocompound metabolism and C5-branced dibasic acid metabolism (Fig. 2, Table S3). The acetolactate synthase that catalyzes the first step of BCAAs biosynthesis was down-regulated by 91.03 folds (Table S1, S3), which is in agreement with previous reports ((Richie et al., 2013; Liu et al., 2015). *LeuABCD*, an operon for leucine biosynthesis, were also down-regulated (Table S1, S3). These data contribute to a more comprehensive understanding of the BCAAs regulation in *V. alginolyticus* and supplies more potential targets for herbicides.

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