Food Control 85 (2018) 369–375

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

iTRAQ-based proteomic analysis of the viable but nonculturable state of *Vibrio parahaemolyticus* ATCC 17802 induced by food preservative and low temperature

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ARTICLE INFO

Article history:
Received 4 July 2017
Received in revised form
10 October 2017
Accepted 11 October 2017
Available online 26 October 2017

Keywords:
Vibrio parahaemolyticus
Viable but nonculturable state
Differentially expressed proteins
iTRAQ
Stress

ABSTRACT

Vibrio parahaemolyticus is a significant food-borne pathogen which can cause serious acute gastroenteritis. The viable but nonculturable (VBNC) state is a survival strategy of some bacteria when they are exposed to environmental stresses. In this study, *V. parahaemolyticus* ATCC 17802 was induced into VBNC state by food preservative at low temperature and oligotrophic condition, and the aim of the study is to compare differentially expressed proteins (DEPs) between the VBNC state and the exponential phase of *V. parahaemolyticus* ATCC 17802 using isobaric tags for relative and absolute quantitation (iTRAQ) technique. Functional annotations were carried out using protein sequence similarity, GO and KEGG pathway analysis. The results showed that *V. parahaemolyticus* ATCC 17802 was induced into the VBNC state at 4°C in seawater containing 10 mmol/L potassium sorbate within 40 days. A total of 1139 proteins were identified,1088 proteins were quantified. Of the DEPs under the VBNC state, 36 were significantly down-regulated and 15 up-regulated. The remarkable down-regulated proteins were type III secretion host injection and negative regulator protein, polar flagellin protein, ribosomal proteins, multidrug efflux pump component MtrF, and other key components involved in metabolism. The notable up-regulated proteins were mainly focused on transporter proteins, outer membrane protein, ferritin and amino acid synthase. The majorities of the significant DEPs were associated with translation, structural constituent of ribosome, rRNA binding, siderophore transmembrane transporter activity, receptor activity, and bacterial-type flagellum organization. This study contributes to a better understanding of the adaptation mechanism of the VBNC state of *V. parahaemolyticus* under food processing and environmental stresses.

1. Introduction

Vibrio parahaemolyticus, a gram-negative marine bacterium, can be frequently isolated from coastal environments and a variety of seafood such as shrimp, oyster, crab, clam, scallop and marine fish. It is a crucial foodborne pathogen usually responsible for acute gastroenteritis (McLaughlin et al., 2005; Nair et al., 2007). Food poisoning outbreaks caused by *V. parahaemolyticus* have spread all over the world including Asia, America, Europe and Africa. It is becoming the leading cause of bacteria-derived food poisoning in the United States and China (Drake, DePaola, & Jaykus, 2007; Lai, Chen, Lin, Chang, & Wong, 2009; Su & Liu, 2007; Wu, Wen, Ma, Ma, & Chen, 2014; Xu et al., 2014).

In recent years, the viable but non-culturable state (VBNC) of bacteria has raised great concern and has been of food safety and public health signification (Li, Mendis, Trigui, Oliver, & Faucher, 2014; Oliver, 2010). VBNC bacteria are those which are viable and maintain metabolic activity and potential pathogenicity but are unable to form colonies on conventional growth media (Oliver, 2005). This state is a survival strategy adopted by bacteria as a response to various environmental stresses and it would resuscitate under favorable conditions. Quite a few studies have indicated that *V. parahaemolyticus* can enter into a VBNC state under unfavorable environmental conditions (Chen, Jane, Chen, & Wong, 2009; Coutard et al., 2007; Lai, Chen, Lin,Chang, &Wong, 2009; Mizunoe, Wai, Ishikawa, Takade, & Yoshida, 2000; Su, Jane, &

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https://doi.org/10.1016/j.foodcont.2017.10.011
0956-7135/

Wong, 2013; Wong, 2004), but the underlying mechanism, the metabolic responses, protein profiles or genetic control of the VBNC state have not been clarified.

The proteomic method has been employed to study the VBNC state of *Escherichia coli* (Asakura et al., 2008; Muela et al., 2008; Zhao et al., 2016), *Enterococcus faecalis* (Heim, Lleo, Bonato, Guzman, & Canepari, 2002), and *Vibrio harveyi* (Jia et al., 2013; Parada et al., 2016). As for the proteomic study on VBNC state of *V. parahaemolyticus*, Lai et al. (Lai et al., 2009) induced the VBNC state of *V. parahaemolyticus* by prolonged incubation at 4 °C in a nutrient-limited medium (MMS-0.5% NaCl), then studied protein profiles by two dimensional polyacrylamide gel electrophoresis (2D-PAGE), and identified 13 up-regulated proteins by mass spectrometry (MS). In recent years, isobaric tags for relative and absolute quantification (iTRAQ), a global protein profiling strategy has emerged as a powerful high-throughput proteomics method (Wright, Noirel, Ow, & Fazeli, 2012), but there is no iTRAQ-based research report on the VBNC state of *V. parahaemolyticus*. In the present study, we induced VBNC state of *V. parahaemolyticus* ATCC 17802 by potassium sorbate and low temperature for the first time, and analyzed the global protein profiles between the VBNC cells and the exponentially growing cells using iTRAQ. The up-regulated and down-regulated proteins were identified, and the functional interpretation of the differentially expressed proteins (DEPs) was conducted by GO annotation and KEGG pathway analysis. This work would be useful to extend our understanding about the molecular mechanism underlying the VBNC state of *V. parahaemolyticus*.

2. Materials and methods

2.1. Bacterial strain and culture conditions

V. parahaemolyticus ATCC17802 was obtained from Guangdong Culture Collection Centre of Microbiology (Guangdong, China). The strain was preserved in 10% (w/v) glycerol broth at −80 °C and was refreshed three times on tryptic soy agar (TSA; BD Diagnostic Systems, Sparks, MD, USA) plates containing 3.0% NaCl at 37 °C. Then the strain was inoculated into tryptic soy broth (TSB) containing 3.0% NaCl and was cultured at 30 °C for 16 h using a shaker incubator set at 120 rpm.

2.2. Induction of the VBNC state of V. parahaemolyticus

After being cultured overnight, the cells were rinsed 3 times with 3% NaCl solutions, and then added into a 3% NaCl solution containing 10 mmol/L potassium sorbate at a cell density of 1.0×10^8 CFU/mL. The samples were stored at 4 °C. At each designated time points, the culturability analysis of the cells was conducted by the plate count method, and the viability was determined using the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, Carlsbad, CA). The stained cells were observed under the fluorescence microscope (Olympus BX51, Olympus, Japan). All the experiments were carried out in triplicate. The cells were induced to enter the VBNC state when there was no colony on plates, but there were viable cells observed green under fluorescent microscope. Then the cells were harvested by centrifugation at 10,000 rpm for 5 min, rinsed two times with PBS (0.1 M, pH 7.2). 10^7 cells were harvested for the following analysis.

2.3. Protein extraction, two-dimensional electrophoresis, digestion and labeling with iTRAQ reagents

The proteins were extracted by acetone extraction method, and the concentrations were determined by a Bradford protein assay kit (Bio-Rad, USA), based on method described by Bradford (Bradford, 1976). After two-dimensional electrophoresis, the DEPs were digested with modified trypsin (Promega, Madison, WI, USA) with the mass ratio of protein: trypsin (50:1) at 37 °C for 16 h. The digested peptides were labeled with iTRAQ reagents according to the protocol of manufacturer (AB Sciex, Foster City, CA).

2.4. Chromatographic separation and MS/MS analysis

First, the labeled peptides were passed through a strong-cation exchange (SCX) column (AB Sciex, Framingham, MA) and desalted by Sep-Pak solid-phase extraction (SPE) cartridges (Waters, Milford, MA) for subsequent reverse phase separation. The peptides were reconstituted in 10 mM ammonium formate (NH_4HCO_2, pH 10, in 2% CAN) and loaded onto an XTerra MS C18 column (3.5 μm, 2.1 × 150 mm, Waters) with 10 mM NH_4HCO_2 as buffer A and 90% ACN/10 mM NH_4HCO_2 as buffer B. The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer A to 8% buffer B for 5 min, 8–18% buffer B for 35 min, 18–35% buffer B for 20 min, and 35–95% buffer B for 2 min. The system was then maintained at 100% buffer B for 4 min before equilibrating with buffer A for 10 min prior to the next sample loading. The peptides were subjected to nano LC-MS/MS analysis using a LTQ-Orbitrap Velos (Thermo Fisher Scientific, San Jose, CA).

2.5. Bioinformatic analysis

The protein sequences of specified species were downloaded from UniProt database (http://www.uniprot.org/). The raw data from mass spectrometer were submitted to Mascot (http://www.matrixscience.com/) for database searching. The results of database searching were subjected to quality control analysis through observing three measurements: length and mass error distribution of identified peptides, the relationship between coverage and mass of identified proteins. If failed to pass quality control, the subsequent analysis aborted, and the experiments would be repeated until passing through our rigid quality control.

For qualitative experiments, identified proteins were subjected to the domain, GO and KEGG annotation. As for domain annotation, we directly mapped identified proteins to InterPro domain database (http://www.ebi.ac.uk/interpro/), which is a powerful integrated functional analysis database of proteins by classifying proteins into families and predicting domains and important sites. GO annotations was used to annotate identified proteins by simple database mapping against UniProt-GOA database (http://www.ebi.ac.uk/GOA). Before mapping, protein ID of identified proteins was firstly mapped to standard UniProt ID of proteins. If a protein has no the corresponding UniProt ID in UniProt database, then InterProScan (http://www.ebi.ac.uk/interpro/) would be used to predict GO annotation of this protein based on protein sequence alignment method. For KEGG pathway annotation, the amino acid sequences of identified proteins were submitted to KEGG automatic annotation server (KAAS) (http://www.genome.jp/kaas-bin/kaas_main), thus obtained the mapping relationship between protein ID and KO identifiers. Then KO identifiers were uploaded to KEGG PATHWAY/BRITE/MODULE mapping tools (KEGG Mapper) (http://www.genome.jp/kegg/tool/map_pathway2.html) for obtaining pathway information.

For quantitative experiments, we identified differential expression proteins (DEPs) in the test and control samples, the fold change of each protein was calculated based on $\log_2$ value of the relative expression between test and control samples. The fold change >1 and p-value < 0.05 were used as the threshold to define the significance of protein expression difference. Then DEPs were further subject to GO and KEGG pathway analysis.