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Comparative proteomic analysis of phenol degradation process by *Arthrobacter*

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ABSTRACT

Phenol is a widespread environmental pollutant due to their broad usage and applications, and presence in many industrial effluents. To date, bacterial degradation of phenol remains to be the preferred method for its removal and remediation. Although the degradation pathway has been extensively studied, the variations in the level of expression of the key enzymes during catabolism are still not quantitatively understood. An explicit quantitative expression helps us know the degradation process in detail. It is important and valuable for us to further understand mechanisms underlying phenol degradation. In this study, we used iTRAQ-based comparative proteomics analysis approach to determine variations in expression and regulation of key enzymes in *Arthrobacter* during phenol degradation. We propose that the phenol biodegradation pathway is mainly determined by 5 pivotal enzymes, which belonged to 3-oxoadipate pathway and tricarboxylic acid cycle. *Arthrobacter* mainly degrades phenol through 3-oxoadipate pathway, which makes the pathway of this strain for degrading the toxic compound clearer. These findings provide new insights into phenol biodegradation process and would help us understand the step by step stages of this metabolic pathway.

1. Introduction

Phenol is a biologically carcinogenic, teratogenic and highly toxic substance found widespread in industrial wastewater. Currently, biological methods are the most used approach in the treatment of wastewater containing this toxicant as they are fast and cost-efficient without any potentials for secondary pollution. Studies on microorganisms involved in phenol degradation have started as early as 1908. Some of the known bacteria that could degrade phenol include *Acinetobacter, Arthrobacter, Pseudomonas, Rhodococcus, Penicillium* and *Candida mycoderm*, and have been widely used in wastewater treatments (Ma et al., 2013; Barbosa et al., 1996; Banerjee and Ghoshal, 2011; Wang et al., 2009; Fialová et al., 2004). Then, in the late 1960's, characterization and elucidation of the mechanisms underlying phenol degradation started, which in later years along with the developments in molecular biology became helpful in improving the degradation ability of bacterial systems (Zídková et al., 2013).

The metabolic pathway of phenol was finally elucidated by Feist and Hegeman (1969) using *Fluorescent Pseudomonads* as the model organisms. However, the limited detection threshold of the methods they used that time like absorbance, oxidation reduction and direct measurement of the suspected enzymes involved, resulted to very low accuracy. Scientific advances and technological developments later allowed Yang and Lee (2007) using polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) to determine the phenol degradation pathway by targeting the genes of interests. Hoyos-Hernandez et al. (2014) later used more sensitive techniques such as radioactive labeling in combination with gas chromatography-isotopes ratio mass spectrometry (GC–IRMS) to detect the metabolic destination of carbons in phenol. Although this method allowed researchers to observe the metabolic process of phenol degradation by following the carbon isotope tracers, to this date, nothing is still known about the function of corresponding key and other related enzymes. Knowledge on the bacterial strains capable of phenol degradation are way behind compared to the process itself. Also, the effect of phenol on

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the bacteria has not yet been thoroughly understood.

High-throughput "omics" methods have been widely applied in different types of biological research. Proteomics, a branch dealing with protein expression, post/translational modification and interaction, were recently used to investigate the phenotypes and metabolism of microorganisms at the protein level. In recent years, progress in the "omics" approaches along with the development of protein separation techniques like 2D gel electrophoresis coupled with MALDI-TOF-MS, provided more information on the total protein content expressed by microorganisms. However, due to its technical complexity and high workload-demanding data analysis, quantification could not be precisely done. Isobaric tagging, a strategy for relative and absolute quantitation (iTRAQ) based on isotope labeling coupled with HPLC-MS for the identification and relative quantification of proteins, could label-quantify 500–600 proteins and analyze their expression variation among several samples. Thus, iTRAQ method could provide a sensitive quantification, quick reaction, complete labeling, quantification and treatment of various samples simultaneously.

Detailed understanding of the metabolic pathway is important in constructing improved phenol degraders and test their bioremediation potentials. Here, we used the *Arthrobacter* to gain more insights into changes in enzymes expressions as a response to phenol enrichment by using iTRAQ strategy to analyze the proteomic variation at different time points. We also investigated the mechanisms underlying phenol metabolism, and inferred its potential phenol degradation pathway.

2. Materials and methods

2.1. Bacteria and cultivation conditions

The bacterium we used was enriched and later isolated from an effluent (Shenhua, Ordos, China). Preliminary characterization showed that it was a rapidly growing, phenol-degrading microbe identified as a strain of *Arthrobacter* (Accession No. KT369868) based on 16s rRNA gene sequence (Supplemental Material). To investigate its degrading activity, the *Arthrobacter* strain was subcultured for two generations in liquid broth media with phenol (0.5 g l^{-1}) to induce the expression of proteins related to phenol degradation. Specifically, the strain was cultivated in liquid media containing phenol (0.5 g l^{-1}) as the only carbon source, supplanted with 1 g l^{-1} NH_4SO_4, 0.5 g l^{-1} $NaNO_3$, 0.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} K_2HPO_4, and 0.2 g l^{-1} NaCl. The inoculum size was set at 5% (v/v). Cells were incubated at 35 °C with agitation (150 rpm) for 120 h. Bacterial concentration was measured by spectrophotometer at 660 nm every 6 h. The bacterial culture was centrifuged at 10,000 × *g* for 10 min at 4 °C. Phenol degradation or lack of thereof was monitored by measuring the remaining phenol in the media using 4-aminoantipyrine method following Ettinger et al. (1951). The degradation ratio was determined based on the absorbance at 510 nm, and the residual ratio was obtained by comparing with the initial value. Samples collected during the log-phase of bacteria cultivated in media without phenol was used as the negative control (sample 1). For the treatments, sampling was carried out three times at the different stages of bacterial growth curve, namely at log-phase, equilibrium state and after the 5-day incubation period (samples 2, 3 and 4). All the samples were collected in triplicates by centrifugation, and the bacterial pellets were stored for downstream analyses.

2.2. Protein preparation and digestion

The bacterial pellets were grinded in liquid nitrogen, resuspended in 0.5 ml lysis buffer containing 8 M urea, 4% m/v 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 40 mM Tris-HCl (pH = 7.0) and 1 mM Phenylmethanesulfonyl fluoride (PMSF) and transferred into a 1.5 mL microcentrifuge tube. Then, 5 μl 1 mM DTT was added 5 min after centrifugation. The samples were ultrasonicated for 6–10 s 8 times and cooled by ice at intervals (Lin et al., 2013). Consequently, samples were kept at 4 °C for 2 h, centrifuged at 13,000 × *g* for 20 min. This was done for each replicate and then mixed. Protein concentration in the supernatant was measured using BCA kit (KeyGEN Biotech. China) according to the manufacturer's instructions and ultramicrospectrophotometer (Q500, Quawell, USA). Protein extracts were sub-sampled for 100 μg (Rydzak et al., 2012) of protein and four times volume of ice-cold acetone and dithiothreitol (DTT) were added to a final concentration of 30 mM and precipitated overnight at −20 °C. Then the precipitate was dried by vacuum evaporator to yield the final protein samples (Wase et al., 2014). A total of 20 μl buffer and 1 μl denaturant were added into the protein samples, mixed and 2 μl reducer was further added, vortexed and collected by low speed centrifugation. The samples were again incubated at 60 °C for 1 h, then 1 μl cysteine blocking agent was added, vortexed, collected by low-speed centrifugation and incubated at room temperature for 10 min. Lastly, 20 μl of 0.25 μg μl^{-1} trypsin (Promega, USA) was added into the treated samples, mixed and digested overnight at 37 °C. The digested peptide samples were collected by low-speed centrifugation.

2.3. iTRAQ sample labeling

The iTRAQ reagent was brought to room temperature (RT) and centrifuged briefly to bring the substance to the bottom of the tube. Then, 70 μl ethanol (95%) was added to every iTRAQ reagent, vortexed and briefly spun down. The iTRAQ reagents were then separately added into the protein samples for tagging, before being incubated at RT for 1 h. The four samples were mixed into the same centrifuge tube, dried by vacuum evaporator, dissolved by 5% acetonitrile and 0.1% formic acid to a final concentration of 1 μg μl^{-1}. MALDI-TOF-MS was used for the detection, and labeling was verified by mass spectrometry particularly of the reported genes.

2.4. On-line Nano-LC and mass spectrometry analysis

The labelled samples were analyzed three times by nano-LC (Eksigent, USA) coupled with Triple TOF 5600 MS (AB SCIEX, USA). The peptides were separated using the C_{18}-CL-120 column (0.075 × 150 mm, Eksigent) and 5%–60% gradient concentration of mobile phase (mobile phase A was 2% acetonitrile in water and 0.1% acetic acid; mobile phase B was 2% water in acetonitrile and 0.1% acetic acid) at a flow rate of 300 nl min^{-1}. The MS detection range was set between 70 and 2500 m z^{-1}, and the precursor ion fragments were analyzed by MS/MS. The three MS data were aggregated and then analyzed.

Protein identification and quantification were conducted in DataAnalysis software (Bruker, Germany). Database search was performed using Mascot server (http://www.matrixscience.com/) with the threshold significance set at $p < 0.05$. We created an in-house database with protein sequences of all the *Arthrobacter* species downloaded from the NCBI database. Search parameters were set as follows: trypsin as the cleavage enzyme, one maximum missed cleavage, cysteine modification, and the mass error tolerance for precursor ions and fragment ions were set to 0.5 D and 0.1 D respectively. In addition, the proteins were quantified by "iTRAQ 4-plex quantification". The identification, classification and prediction of proteins were performed using INTERPRO (http://www.ebi.ac.uk/interpro/scan.html), COG (http://weizhong-lab.ucsd.edu/