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Quantitative proteomic analysis reveals that chemotaxis is involved in chlortetracycline resistance of *Aeromonas hydrophila*

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ABSTRACT

In recent years, *Aeromonas hydrophila*, which has been classified as a food borne pathogen, has presented with increased levels of antibiotic resistance, with the mechanisms of this resistance being poorly understood. In this study, iTRAQ coupled mass spectrometry was employed to compare differentially expressed proteins in chlortetracycline (CTC) resistant *A. hydrophila* relative to a control strain. Result showed that a total of 234 differential proteins including 151 down-regulated and 83 up-regulated were identified in chlortetracycline resistance strain. Bioinformatics analysis showed that chemotaxis related proteins, such as CheA-2, CheR-3, CheW-2, EnvZ, PolA, FliS and FliG were down-regulated in addition to previously reported tricarboxylic acid cycle (TCA) related proteins also being down-regulated. A subset of identified differentially expressed proteins was then further validated via Western blotting. Exogenous metabolite combined with CTC further enhanced the bacterial susceptibilities to CTC in *A. hydrophila*. Furthermore, a bacterial survival capability assay showed that several chemotaxis related mutants, such as *ΔcheR-3* and *ΔAHA_0305*, may affect the antimicrobial susceptibility of *A. hydrophila*. Overall, these findings contribute to a further understanding of the mechanism of CTC resistance in *A. hydrophila* and may contribute to the development of more effective future treatments.

Biological significance: A. hydrophila is a well-known fish pathogenic bacterium and has presented with increasing levels of antibiotic resistance, with the mechanisms of this resistance being poorly understood. Our current study compared the differentially expression proteins between chlortetracycline (CTC) resistant and control stains via an iTARQ-based quantitative proteomics method. Chemotaxis related proteins were down-regulated in CTC resistant strain but exogenous metabolite addition increased bacterial susceptibility in *A.hydrophila*. Significantly, chemotaxis related genes depletion affected antimicrobial susceptibilities of *A.hydrophila* indicating the role of chemotaxis process in antibiotics resistance.

1. Introduction

As worldwide seafood consumption increases, the number of bacterial disease outbreaks has also been on the rise and has contributed to billions of dollars of lost revenue. Of these pathogens, *Aeromonas hydrophila*, a well-known fish pathogenic bacterium, is primarily found in temperate and freshwater environments and causes opportunistic infections in organisms like wild carp, shellfish, grass carp and shrimp [1,2]. Therefore, *A. hydrophila* has been classified as a potential food borne pathogen and has aroused attention by food safety organizations [3]. In recent years, tons of antibiotics have been dumped into pathogen prone environments to reduce pathogenic bacterial loads. However, this approach has instead increased the presence of multidrug-resistant strains, including *A. hydrophila*, that pose a serious threat to the aquaculture industry and public health [4,5]. When treating aquatic environments, tetracyclines, which include the monocyclines doxycycline and chlortetracycline (CTC), are commonly used and have shown efficacy against gram-positive and gram-negative bacteria. Of these antibiotics, CTC is one of the most widely used in aquaculture and acts by preventing aminoacyl-tRNA binding of the ribosomal acceptor (A) site, thereby blocking protein synthesis [6]. Thus, a systemic understanding the mechanisms of bacterial CTC resistance is urgently needed to aid in the prevention and control of these aquatic pathogens.

Classically, tetracycline antibiotic resistance involves an active efflux pump, a ribosomal mutation or another ribosomal protective

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mechanism, thus making the antibiotic functionless [7–12]. In fish farms, tetracycline resistance is commonly mediated by either the *tet* family of proton-dependent efflux pumps or by ribosomal protection via cytoplasmic proteins that are mainly found in gram–negative bacteria [13,14]. However, antibiotic resistance is a complicated phenomenon; with it seeming likely that resistance mechanism would involve comprehensive networks, such as genes, proteins and several biological processes. For example, our previous research illustrated the importance of the fatty acid biosynthesis pathway in tetracycline resistance in *A. hydrophila* biofilm, thus further indicating that the intrinsic tetracycline resistance mechanisms remain largely elusive [15].

To further characterize the mechanisms of antibiotic resistance, state-of-the-art proteomics strategies have be applied [16]. For example, one such approach was used to identify differential expression associated with oxytetracycline (OXY) resistance in *Edwardsiella tarda* and identified various biological processes, such as ABC transport, and implicated the central metabolic pathway and translation related proteins [17]. Additionally, Lin et al. showed that in *Bacillus subtilis*, chloramphenicol, erythromycin and gentamycin affect carbon metabolism and transport/binding related proteins [18]. Due to the fact that proteins serve as functional building blocks and are implicated in many complicated mechanisms pertaining to antibiotic resistance, this study utilized a proteomic approach to identify differentially expressed proteins in CTC resistant *A. hydrophila* (*Ah*-CTC-R) relative to a sensitive control strain (*Ah*-CK). These findings were then analyzed using bioinformatics and multiple pathways and a number of biological processes were implicated to play a role in antibiotic resistance. A subset of the identified differentially expressed proteins was then further substantiated by Western blot. Furthermore, several related gene mutants were constructed and their antimicrobial susceptibilities were evaluated using a survival capability assay. Overall, this research provides a novel understanding of the mechanisms of antibiotic resistance in *A. hydrophila*.

2. Materials and methods

2.1. Bacterial strains and sample preparation

A. hydrophila (strain ATCC 7966) was cultured overnight in Luria-Bertani (LB) medium at 30 °C on a shaker at 200 rpm. The *Ah*-CTC-R strain was selected from the original *A. hydrophila* (*Ah*-CK) strain using CTC-induction as previously described [19]. The minimum inhibitory concentrations (MICs) of *Ah*-CK and *Ah*-CTC-R to CTC were determined to be 40 and 640 μg/mL respectively. Culture densities were determined at 600 nm with a UV spectrophotometer (Varian, Palo Alto, CA, USA). Proteins were extracted from each of the strains after 3 h incubation and were harvested by centrifugation (10,000 rpm, 10 min, and 4 °C). The pellets were washed three times with PBS before being resuspended in dissolution buffer (8 M urea, 0.1% SDS and 1% Triton-X 100 in 0.2 M TEAB). The whole cell proteins were isolated by centrifuging at 12,000*g* for 30 min at 4 °C, with the supernatants stored at − 80 °C until further use. Protein concentrations were determined via Bradford assay.

2.2. Digestion and iTRAQ labeling

Each protein sample (50 μg each) was reduced with 50 mM TCEP, alkylated by 200 mM methyl methylthiomethyl sulfoxide (MMTS), and digested with trypsin at a ratio of 1:50 as previously described [20]. The digested peptides were labeled using isobaric tags for relative and absolute quantitation (iTRAQ) kits (AB SCIEX, Framingham, MA, USA) according to the manufacturer's protocols. The labeled peptide samples were divided into two biological repeats for the *Ah*-CK (113 and 117) and the *Ah*-CTC-R (115 and 119) treatments. Labeled peptides were then pooled, desalted using a Sep-Pak Vac C18 Column (Waters Inc., Milford, MA) and dried using a CentriVap concentrator (Labconco Inc., Kansas City, MO).

2.3. Quantitative analysis using LC–MS/MS

The iTRAQ-labeled peptides were resuspended in 0.1% FA (formic acid) and analyzed on an AB Sciex TripleTOF 5600 mass spectrometer (AB SCIEX; Concord, ON, Canada) with a NanoAcquity UPLC (Waters, Milford, MA, USA) system as previously described [21]. The generated data was analyzed using ProteinPilot version 4.5 software. The *A. hydrophila* ATCC 7966 dataset was download from the Uniport database on Feb. 1st, 2017 and searched using the following parameters: cysteine carbamidomethylation and iTRAQ labeling as fixed modifications, methionine oxidation as a variable modification and digestion by trypsin with at least two missed cleavages. The identified proteins with at least two peptide matches and a confidence threshold > 99% were further analyzed. Proteins were determined to be differentially expressed if a minimum of a 2-fold change was noted and if a p-value < 0.05 was obtained.

2.4. Bioinformatics analysis

GO annotations and KEGG pathway analysis of altered proteins were analyzed with OmicsBean (http://www.omicsbean.cn/) [22]. Protein functions were categorized according to the assigned GO annotations, which were based on biological process hierarchy. Furthermore, protein-protein interactions were predicted and KEGG pathway analysis was performed using STRING 10.5 and displayed using Cytoscape 3.5.1 [23,24].

2.5. Western blotting

Western blotting experiments were performed as previously described [25]. *Ah*-CK and *Ah*-CTC-R protein samples (~20 μg) were run on 12% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA) for 30 min at 1.3 mA using a Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The membrane was blocked in 5% skim milk with PBS buffer containing 0.05% Tween 20 (PBST) for 1 h and then probed with primary antibody (1:4000) and allowed to incubate overnight at 4 °C. After washing five times with PBST for 5 min each, the membrane was incubated with HRP goat anti-mouse IgG secondary antibody (1:4000) and incubated for 1 h at room temperature. The membrane was then washed three times in PBST, visualized using the ECL system (Bio-Rad, Hercules, CA, USA), and imaged with a ChemiDoc™ MP (Bio-Rad, Hercules, CA, USA).

2.6. Construction of deletion strains

In this experiment, two strains of *Escherichia coli* MC1061 (λpir) and S17-1 (λpir) with suicide plasmid pRE112 were utilized to generate the deletion mutants by *sacB* suicide gene-based allelic exchange [26]. First, primers were designed and gene cloning upstream and downstream of an approximately 500 bp fragment from *Aeromonas hydrophila* ATCC 7966 was performed. The obtained fragments were then cloned into a pRE112 plasmid using a ClonExpress® MultiS one-step cloning kit (Vazyme). Next, *E. coli* MC1061 competent cells were transformed with the recombinant suicide plasmids. The recombinant plasmid was then extracted and transformed into *E. coli* S17-1 competent cells. The recombinant plasmid construct was verified by sequencing and conjugated into wild-type *A. hydrophila* using *E. coli* S17–1 as the donor strain. Integration of the plasmid into the chromosome was inoculated to LB plates containing chloramphenicol (30 μg/mL) and ampicillin (100 μg/mL). Next, chloramphenicol and ampicillin resistant *A. hydrophila* transformants were cultured on LB plates supplemented with 20% sucrose to isolate colonies that were sucrose resistant, with sucrose resistant colonies also tested for chloramphenicol sensitivity to ensure loss of the suicide plasmid. Deletion mutants were verified by