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N-acetylglucosamine enhances survival ability of tilapias infected by *Streptococcus iniae*

Zhi-xue Cheng [1], Yan-mei Ma [1], Hui Li*, Xuan-xian Peng*

Center for Proteomics and Metabolomics, State Key Laboratory of Bio-Control, MOE Key Lab Aquatic Food Safety, School of Life Sciences, Sun Yat-sen University, University City, Guangzhou 510006, People's Republic of China

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ABSTRACT

Streptococcus iniae infection has emerged as a serious fish health and economic problem in the global aquaculture operations. Current antibiotic options are few and possess severe practical limitations and potential adverse environmental impacts. The major factor contributing to the large burden of *S. iniae* disease in aquaculture is the lack of fundamental knowledge of innate immunity against the pathogen. In the present study, we use a tilapia model to explore which metabolites are crucial for the defense against the infection caused by *S. iniae*. We establish GC/MS based metabolic profile of tilapia liver and then compare the metabolic difference between survivals and the dying fish post the bacterial infection. We identify elevating *N*-acetylglucosamine in survival group as the most crucial metabolite differentiating the survivals from the dying in these fish infected by *S. iniae*. Exogenous *N*-acetylglucosamine significantly elevates survival ability of tilapia against the infection caused by *S. iniae*. Our findings highlight the importance of metabolic strategy against bacterial infections.

1. Introduction

Streptococcus iniae is a beta-hemolytic, Gram-positive, sphere-shaped bacterium belonging to the genus *Streptococcus*. This bacterium has a broad host range and affects at least 27 species of fishes, including a large number of economically important species such as rainbow trout, tilapia, sea bass, channel catfish, barramundi and Japanese flounder [1–6]. Infections in fish manifest as meningoencephalitis, skin lesions and septicemia, and are highly lethal with 30–50% mortality in outbreaks. *S. iniae* has emerged as a leading fish pathogen and caused substantial economic losses in the aquaculture industry worldwide [7]. Control of the infections caused by *S. iniae* has become an important issue for fish health aquaculture.

Immunoprophylaxis has been an effective measure to maintain a sustainable aquaculture [8–11], but only experimental *S. iniae* vaccines in the forms of subunit vaccine [12,13], DNA vaccine [14], and attenuated live vaccine [15–18] have been reported. In this background, it is especially important to cope with the pathogen through regulation of innate immunity.

Recently, metabolomics has been the growing field. It detects and quantifies the low molecular weight molecules produced by active, living cells in their life cycles. Metabonomics investigates how the metabolic profile of a complex biological system changes in response to stresses like disease, toxic exposure, or dietary change [19–21]. Metabolomics speed up an understanding of global metabolic characteristics and identification of metabolic biomarkers [19,22]. Very recently, we have revealed variation of metabolomes separately responsible for survivals and death of crucian carps infected by *Edwardsiella tarda*, in which unsaturated fatty acid biosynthesis and fructose and mannose metabolism are identified as the most key pathways and elevating palmitic acid and decreasing D-mannose are characterized as the most crucial metabolites [23]. This motivated us to investigate differential metabolomes in response to *S. iniae* infection, and then identified crucial metabolites contributed to the varied metabolomes. Furthermore, we wanted to exploit a metabolite to identify, among the crucial differential metabolites, a potential modulator capable of elevating survivals in the tilapias infected with the bacterial pathogen. The results are reported as follows.

* Corresponding authors. State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, University City, Guangzhou 510006, People's Republic of China. Fax: +86 20 8403 6215.
E-mail addresses: lihui32@sysu.edu.cn (H. Li), pxuanx@sysu.edu.cn, wangpeng@xmu.edu.cn (X.-x. Peng).
[1] The first two authors are equally contributed.

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2. Materials and methods

2.1. Chemicals and fish

The chemicals used in this study were obtained from Sigma–Aldrich. Tilapias were bought from a local fish farm (Panyu, Guangzhou) with 5 cm ± 0.5 cm length and 1.8 g ± 0.2 g body weight and acclimated in stock tanks (80 × 75 × 90 cm) for 1 week. The fish were fed with commercial pelleted feed twice a day.

2.2. Bacterial strain and infectious symptoms

S. iniae, a gift from Professor Anxin Li, School of Life Sciences, Sun Yat-sen University, were cultured in Brain Heart Infusion Broth (BHI) for 36 h at 22 °C, then centrifuged and diluted using saline solution until 1.0 of OD_{600} for follow-up test. LD_{50} dose used for challenge was determined by intraperitoneal injection into tilapias fed at 30 °C. In brief, 10 fish for each dose were challenged with 5-fold dilution series of doses ranging from 2×10^4 to 5×10^5 CFU/fish for intraperitoneal injection. The LD_{50} used was 5×10^4 CFU/fish. Some of the fish started to show infectious symptoms after 40 h and died within 60 h. The remaining infected fish survived without visible symptoms.

2.3. Tilapia samples infected by S. iniae

After one week of acclimation, tilapias were randomly divided into control and two test groups, 20, 30 and 30 fish, respectively. While the control group was injected with sterile saline, the two test groups were intraperitoneal infected with 5×10^4 CFU/fish separately for preparation of GC/MS sample (test group 1) or investigation of survival rate (test group II). Fishes with and without infectious symptoms were separately collected at 48 h in test group 1, designed as the dying and survival groups, ten for each group, respectively. Meanwhile, ten fishes were randomly collected from control. Livers were isolated from these animals and used for GC/MS analysis.

2.4. Sample preparation for GC–MS

Sample preparation was performed as described previously [24,25]. In brief, livers from 10 fish were obtained and appropriate volume of cold methanol was added according to the weight (800 μL/100 mg). After centrifugation at 120,000× *g*, 4 °C, 50 μL supernatant was collected. The supernatant containing 10 μL 0.2 mg/mL ribitol (Sigma) as an analytical internal standard was concentrated in a rotary vacuum centrifuge device (LABCONCO). The dried extracts were used for GC/MS analysis.

2.5. GC/MS analysis

GC–MS analysis was carried out with a variation on the two-stage technique [26]. In brief, samples were derivatized and then used to firstly protect carbonyl moieties through methoximation, through a 90 min, 37 °C reaction with 80 μL of 20 mg/mL methoxyamine hydrochloride (Sigma–Aldrich) in pyridine, followed by derivatization of acidic protons through a 30 min reaction with the addition of 80 μL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Sigma–Aldrich) at 37 °C. The derivatized sample of 1 μL was injected into a 30 m × 250 μm i.d. × 0.25 μm DBS-MS column using splitless injection and analysis was carried out by Trace DSQ II (Thermo Scientific). The initial temperature of the GC oven was held at 85 °C for 5 min followed by an increase to 270 °C at a rate of 15 °C min^{-1} and then held for 5 min. Electron impact ionization (EI) mode was selected and ionization energy was 70 eV. Helium was used as carrier gas and flow was kept constant at 1 mL min^{-1}. The MS was operated in a range of 50–600 m/z. Two technical replicates were prepared for each sample.

2.6. Data processing for GC/MS

2.6.1. Data processing

Spectral deconvolution and calibration were performed using AMDIS and internal standards. A retention time (RT) correction was performed for all the samples, and then the RT was used as reference against which the remaining spectra were queried and a file containing the abundance information for each metabolite in all the samples was assembled. Metabolites from the GC–MS spectra were identified by searching in National Institute of Standards and Technology (NIST 08) Mass Spectral Library. Among the detected peaks of all the chromatograms, 187 peaks were considered as endogenous metabolites excluded internal standard ribitol. The resulting data matrix was normalized by the concentrations of added internal standards and the total intensity. Normalized peak intensities formed a single matrix with Rt-m/z pairs for each file in the dataset. This file was then used for subsequent statistical analyses.

2.6.2. Statistical analyses

Metabolites subtracted the median metabolites and were scaled by the quartile range in the sample. Z-score analysis scaled each metabolite according to a reference distribution [27], and calculated based on the mean and standard deviation of reference sets. Hierarchical Clustering was performed on the log transformed normalize date, completed in the R platform with the package g plots (http://cran.r-project.org/src/contrib/Descriptions/gplots.html) using the distance matrix.

2.6.3. Pattern recognition

Multivariate statistical analysis included principal component analysis (PCA) (SIMCA-P (Umetics)). PCA was used to discriminate sample patterns, to identify the metabolites associated with temperature and to minimize the inter-individual variation's influence. SPSS 13.0 and Prism v5.01 (GraphPad, La Jolla, CA, USA) were used to draw the histogram and the scatter plot.

2.7. Exogenous addition of N-acetylglucosamine and bacterial challenge

Fish fed at 30 °C were randomly divided into control, test I, test II and test III groups, 20 fish for each group. Control fish were intraperitoneally injected with 20 μL sterile saline. Fish in test I, test II and test III were injected 0.5 mg, 1 mg and 2 mg *N*-acetylglucosamine which was dissolved in 20 μL of sterile saline, respectively, once daily for 5 days. These fish were intraperitoneally challenged with *S. iniae* (7×10^4 CFU/fish). The animals were observed twice daily for ten days.

3. Results

3.1. Metabolomic profiling of tilapia liver

To identify the key metabolic pathways and crucial metabolites as biomarkers that were required for the fish survived from bacterial infection, tilapias were exposed to or not exposed to LD50 of *S. iniae* as test group and control groups, respectively. The test group was divided into groups 1 and II for GC/MS analysis and survival investigation, respectively. Since approximately half of fishes died in 60 h and the others survived in ten days in the