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Construction of differentially expressed genes library of bighead carp (*Aristichthys nobilis*) exposed to microcystin-lr using ssh and expression profile of related genes

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ABSTRACT

Microcystins (MCs) are hepatotoxic cyclic heptapeptides produced by cyanobacteria (blue-green algae). There are more than 70 MCs variants of which the most common and widely studied is MC-LR. We screened the hepatocellular differentially expressed genes against MC-LR in the bighead carp (*Aristichthys nobilis*). Suppression subtractive hybridization was used to construct the forward subtracted and reverse subtracted cDNA libraries, and one hundred and thirty two positive clones (seventy one in forward library and sixty one in reverse library) were randomly selected and sequenced. Finally, fifty five reliable sequences from the forward subtracted library were used in a homology search by BLASTn and BLASTx, as were 57 reliable sequences from the reverse subtracted library. Furthermore, eight analyzed sequences from the forward subtracted cDNA library and seven from the reverse subtracted library were found to be non-homologous sequences. The screening identified genes induced by MC-LR in both libraries that are involved in various processes, such as energy metabolism, immunity, and apoptosis. Some are cytoskeleton- and transportation-related genes, while signal transduction-related genes were also found. Significant genes, such as the apoptosis-related gene *p53* and the proto-oncogene *c-myc*, are involved in inhibition of the MC-LR response in the reverse subtracted library. In addition, several immune-related genes, which play an important role in antioxidation and detoxification of MC-LR, were characterized and identified in both of the subtracted libraries. The study provides the basic data to further identify the genes and molecular mechanism of detoxification of microcystins.

1. Introduction

The occurrence of toxic cyanobacterial blooms in eutrophic lakes, reservoirs, and recreational waters has become a worldwide problem, and there is public concern that cyanotoxins that may enter drinking water and pose a threat to public health. Several cyanobacterial species, may produce a variety of potent toxins, including hepatotoxins. Among the hepatotoxins, cyclic peptides known as microcystins (MCs) are the predominant toxins in freshwaters worldwide. The molecular structure of MCs consists seven variable amino acids and the amino acid of prominent note is the nonpolar-linked C_{20} amino acid, (3-amino-9-methoxy-10-phenyl-2, 6, 8-trimethyldeca-4, 6-dienoic acid), abbreviated as ADDA [1]. More than 70 MCs have been isolated and identified to date [2], but only a few have been found in high concentrations. Among the MCs, the most common, and also the most extensively studied, is microcystin-LR (MC-LR). MC-LR can inhibit the phosphatases 1 and 2A (PP1, PP2A) [3], resulting in the disruption of many cellular processes and alteration of cytoskeletal structures [4], signal transduction, and the regulation of enzyme activities [3,5], as well as hepatic hemorrhage. MC-LR can also promote liver tumor formation through inhibition of PP1 and PP2A activities [6–8]. Human illnesses attributed to MC-LR include gastroenteritis and allergic/irritation reactions, MC-LR plays a role in primary liver cancer [9]. Metabolism of MC-LR begins with a conjugation reaction to glutathione (GSH) catalyzed by glutathione *S*-transferases (GST) in plants and animals [10]. Several findings on the antioxidant response to exposure to MC-LR in animals reveal that organisms try to reduce the damage caused by the toxins by using the antioxidant defense system, which comprises both enzymatic and non-enzymatic mechanisms [11–13]. There is a positive correlation of MC-LR-induced oxidative stress leading to hepatic GSH depletion, lipid peroxidation, stress protein HSP-70 expression, and alterations in the activity profile of certain antioxidant enzymes [11]. Most recently, alterations in the expression of genes have been investigated at the level of various genes, such as immune genes and transcriptional regulation genes of mammals exposure to MC-LR [14–16]. The conjugation of MC-LR with GSH catalyzed by GST

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was verified in several mammalian experiments and, moreover, microarrays have been used to characterize and analyze the patterns of gene expression induced by MC-LR in zebrafish [17]. However, this technology is not cost effective for average laboratories. Therefore there is little information on the changes of gene expression in fish feeding on phytoplankton when they are exposed to MC-LR, including the differential expression of detoxification-related genes of bighead carp treated with MC-LR.

Bighead carp (*Aristichthys nobilis*) is one of the most important freshwater phytoplanktivorous fish. The filter feeding fish are especially significant to humans because of their role in aquatic ecosystems as direct consumers of phytoplankton, including large amounts of toxic blue-green algae which may contain high concentrations of MCs, their importance as a food fish, and their potential for biological management of cyanobacterial blooms [18]. Although its natural range is in the lowland rivers and shallow lakes of China [19], since bighead carp has been introduced worldwide, primarily for the purpose of cyanobacterial blooms control in hypereutrophic lakes [20]. Previous studies suggested that in the gut MCs content generally showed a reversed pattern: content was highest in the phytoplanktivorous fish, followed by the omnivorous and carnivorous fish. However, MCs content in the liver was highest in carnivorous, followed by omnivorous fish, and was lowest in phytoplanktivous and herbivorous [21]. Chen et al noted that MCs content in the liver had a strong correlation with that in the muscle of the bighead carp. These suggest that the phytoplanktivorous fish is strongly resistant to MCs than other fishes due to less toxin accumulation in liver, the target organ of toxins [22,23]. So it is essential to study the differentially expressed genes induced by MC-LR to further understand the mechanism of hepatic anti-oxidation and detoxification in phytoplanktivorous fish, especially bighead carp.

Suppression subtractive hybridization (SSH), using isolated mRNA, plays an important role in molecular investigations of interesting functional genes [24]. SSH is used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress non-target DNA amplification. The method is based on the suppression PCR effect, and the technique is simple and efficient for generating cDNA highly enriched for differentially expressed genes of both high and low abundance [25]. In the present study, we have investigated genes expressed in response to MC-LR injection in the liver (the target organ of the toxin) of bighead carp, in order to screen and identify genes involved in defense and detoxification in the phytoplanktivorous fish.

2. Materials and methods

2.1. Bighead carp exposure MC-LR experiment

Bighead carp (100 ± 10 g) were held in aquaria with a continuous system of water filtration and aeration, and the temperature was kept constant (25 ± 2 °C). Fish were not fed and were acclimatized for three days before the beginning of the exposure experiments. One group of four fish individuals was intraperitoneally injected with a dose of 200 μg MC-LR/kg body weight, dissolved in 0.8% sterile saline, and the control group of four fish was injected with 0.8% sterile saline. Four fish in both groups were killed at 0.5, 1, 3 and 5 h post-injection, respectively. Liver samples were dissected out and stored separately for extracting total RNA.

2.2. Total RNA extraction and mRNA isolation

Total RNA was extracted from liver samples weighing about 100 mg using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The mRNA was isolated by using the OligotexTM-dT30 < Super>mRNA Purification Kit (TaKaRa, Japan) according to the manufacturer's instructions. The amount and purity of total RNA and mRNA were estimated by measuring OD at λ 260 nm and λ 280 nm. The integrity of the total RNA was examined by electrophoresis equipped with a 1% agarose/EtBr gel. The mRNA was concentrated with 100% ethanol and 3 mol/L NaAc, before being used as starting material to construct the SSH cDNA library.

2.3. Semi-quantitative RT-PCR of GST gene expression and statistical analysis

A semi-quantitative RT-PCR method was used to measure GST gene expression. Total RNA samples from liver of control group fish and exposure groups (0.5, 1, 3 or 5 h) were applied to reverse transcription PCR. The yield of cDNA was measured according to the PCR signal generated from internal standard house-keeping gene β-actin[1] (primers, Table 1) amplified from 28 cycles. According to the optical density of electrophoretic bands of GST and β-actin[1] gene, the ratios of GST/β-actin[1] were analyzed by using Quantity One® Software.

2.4. SSH cDNA library construction and analysis of subtraction efficiency

According to the semi-quantitative RT-PCR results, mRNA from 0.5 h post-injection sample was selected for reverse transcription into cDNA. Two SSH experiments were performed in parallel using different testers, thereby constructing the forward and reverse subtracted cDNA libraries (FSL and RSL). Subtracted cDNA in the forward library was made from control group mRNA as driver; cDNA made from exposure group mRNA made up the driver of the reverse library. After the synthesis of second strand cDNA, samples were digested with *Rsa*I to generate shorter, blunt-ended, double-stranded cDNA fragments necessary for adaptor ligation. Subtraction was performed in two directions. *Rsa*I-digested tester cDNA was ligated with either Adaptor 1 or Adaptor 2R for use in the subtraction. Adaptor-ligated tester cDNA was denatured and hybridized with excess driver cDNA. Fresh denatured driver DNA was added, and the second hybridization was performed. The samples were PCR amplified: the primary PCR was 27 cycles, and the secondary was 12 cycles. The secondary PCR products were purified using TIANgel Midi Purification Kit (TIANGEN, China). The pMD19-T vector (TaKaRa, Japan) was used to insert screened cDNA fragments and transformed into *Escherichia coli* DH5α-competent cells (TIANGEN, China). Then the transformed cells were plated on the LB/ampicillin/IPTG/X-Gal medium. Plates were incubated at 37 °C overnight to obtain colonies harboring subtractive sequence fragments. Individual recombinant white colonies were randomly picked and cultured in liquid LB agar at 37 °C overnight. Then the

Table 1
Primer sequences used in the semi-quantitative RT-PCR and subtraction efficiency.

Primer	Sequence	Product size (bp)
GST-F	5′-AGAACGGGCTTTGATTGAC-3′	267
GST-R	5′-AAGGTTGACAGTATTGTAGGGA-3′	
β-actin[1]-F	5′-ATTGCCGCACTGGTTGTT-3′	340
β-actin[1]-R	5′-TTTCCCTGTTGGCTTTGG-3′	
β-actin[2]-F	5′-GCCGTGACCTGACTGACTA-3′	275
β-actin[2]-R	5′-ACCGCAAGACTCCATACCC-3′	
M13-F	5′-TGTAAAACGACGGCCCAGT-3′	
M13-R	5′-CAGGAAACAGCTATGACC-3′	

1: semi-quantitative experiment, 2: subtraction efficiency experiment.