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Differentially expressed genes of *Tetrahymena thermophila* in response to tributyltin (TBT) identified by suppression subtractive hybridization and real time quantitative PCR

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Abstract

Tributyltin (TBT) is widely used as antifouling paints, agriculture biocides, and plastic stabilizers around the world, resulting in great pollution problem in aquatic environments. However, it has been short of the biomonitor to detect TBT in freshwater. We constructed the suppression subtractive hybridization library of *Tetrahymena thermophila* exposed to TBT, and screened out 101 Expressed Sequence Tags whose expressions were significantly up- or down-regulated with TBT treatment. From this, a series of genes related to the TBT toxicity were discovered, such as glutathione-*S*-transferase gene (down-regulated), plasma membrane Ca^{2+} ATPase isoforms 3 gene (up-regulated) and NgoA (up-regulated). Furthermore, their expressions under different concentrations of TBT treatment (0.5–40 ppb) were detected by real time fluorescent quantitative PCR. The differentially expressed genes of *T. thermophila* in response to TBT were identified, which provide the basic to make *Tetrahymena* as a sensitive, rapid and convenient TBT biomonitor in freshwater based on rDNA inducible expression system.

Keywords: *Tetrahymena thermophila*; Tributyltin; Suppression subtractive hybridization; Expressed Sequence Tags; Biomonitor

1. Introduction

Butyltin compounds have been extensively used as polyvinyl chloride (PVC) stabilizers, industrial catalysts, agricultural biocides, wood preservatives, and especially as an additive in paint to prevent bio-fouling on ship hulls since the 1960s (Hoch, 2001). Unfortunately, they are highly toxic compounds in aquatic systems when introduced via antifouling agents (Appel, 2004; Konstantinou and Albanis, 2004). Depending on the number of organic moieties, butyltin compounds are classified as mono-, di-, tri-, or tetrabutyltins, and the most toxicity of them is tributyltin (TBT) (Hoch, 2001). TBT poses a significant hazard to aquatic organisms, such as mussels, clams, marine worms, seastars, fishes, through genotoxic, cytotoxic, neurotoxic, hepatotoxic, cutaneous toxicity, teratogenic, immunosuppressive and endocrine disruptive activities (Snoeij et al., 1987; Fent, 1996; Bentivegna and Piatkowski, 1998; Hagger et al., 2002; Békri and Pelletier, 2004; Roepke et al., 2005). Use of antifouling agents containing TBT as their major butyltin components on small ships (<25 m) has been prohibited in most developed countries, but butyltin compounds still persist in aquatic ecosystems, particularly in areas of coastal environments, such as heavy boating, fishing traffic, and docking activities. Furthermore, most developing countries have no restriction on the use of TBT, and the widespread occurrence of TBT has been evidenced in lakes and rivers (Evans et al., 1995; Jiang et al., 2001; Van Wezel and Van Vlaardingen, 2004), so the problem of TBT containment in estuarine and inland water systems becomes more serious from day to day as in marine environment.

In order to monitor the toxicity of TBT in the aquatic ecosystem, several biomonitors have been established, such as the zebra mussel *Dreissena polymorpha* (Regoli et al., 2001; Roper et al., 2001), the clams *Venerupis decussata* and *Mya arenaria* (Gómez-Ariza et al., 2000; Yang et al., 2006), the

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gastropod *Nucella lapillus* (Quintela et al., 2000), and the amphipod *Caprella* spp. (Takeuchi et al., 2001). However, these biomonitors are mainly used in the marine environments, and their exposed period is more than 28 days to detect TBT in tissues of clams accumulated or more than 5 months to assess the phenomenon of imposex in gastropod (Quintela et al., 2000; Yang et al., 2006). Therefore, it is essential to find and establish a new efficient and sensitive biomonitor to forecast TBT in freshwater environments.

As a ubiquitous freshwater ciliated protozoan, *Tetrahymena thermophila* has many characteristics offering particular advantages for aquatic toxicological and ecotoxicological studies (Elliott, 1973; Sauvant et al., 1999; Fu et al., 2005). Analyses of mRNA complexity and recent Expressed Sequence Tags (ESTs) projects have shown that the *Tetrahymena* genome conserves a fairly complete set of ancestral eukaryotic functions and shows a high degree of functional homology with human and mammalian genomes (Fillingham et al., 2002; Turkewitz et al., 2002); moreover the *Tetrahymena* Macronuclear Genome Project has been completed and the *Tetrahymena* Genome Database (TGD; www.ciliate.org) has also been established recently (Eisen et al., 2006; Stover et al., 2006); high-copy-number rDNA vectors and endogenous or well-modified strong promoters have been engineered for the purpose of gene inducible and over-expression in *T. thermophila* (Turkewitz et al., 2002). Therefore, *Tetrahymena* has potential as TBT biomonitor based on rDNA inducible expression system: identifying some differentially expressed genes response to TBT, and inserting their promoter sequences into *Tetrahymena* rDNA vector, and finally making such rDNA expression system as biomarker.

In this study, we looked at differential gene expression induced by TBT treatment of *Tetrahymena* by suppression subtractive hybridization (SSH). This is a highly effective genome-wide approach that enriches for differentially expressed mRNA transcripts by generating subtracted cDNA libraries (Diatchenko et al., 1996; Dilger et al., 2003; Cao et al., 2004; Lu et al., 2005). Differential expression of some of the genes identified by SSH was confirmed by real time quantitative PCR. This method is of greatly sensitive for detection and quantification of gene expression levels, in particular for low abundance mRNA (Pfaffl et al., 2002; Wang and Seed, 2003; Miao et al., 2006).

2. Materials and methods

2.1. Cell culture and preliminary toxicity experiment

Cells of *T. thermophila* were grown axenically at 30 °C in a culture medium containing Tryptone 15 g (Oxoid), yeast extract 5 g (Oxoid) and glucose 1 g/1000 ml (pH 7.0–7.2). The shock solution of TBT was confected by dissolving the accurately weighed tributyltin chloride (90%, Acros Organic) into DMSO (Sigma) to form the concentration of 9 ppm, and stored it at 4 °C (Yang et al., 2006). The shock solution was added to culture medium to final concentrations of 0, 5, 10, 15, 20, and 25 ppb. *T. thermophila* in early stationary phase (2×10^5 cells/ml) were inoculated into the TBT-treated medium and incubated for 24 h. At 5 ppb TBT treatment, the cell density was similar to control; therefore, 5 ppb was chosen as the dose for suppression subtractive hybridization.

2.2. mRNA isolation

Cultures 50 ml in volume were incubated at 30 °C in the presence of 5 ppb TBT (T-TBT, the treatment) or no TBT (T, the control) for 24 h. An average of 1×10^6 cells/ml were harvested from each culture by centrifugation (Beckman Instruments Inc., Fullerton, USA) at 9000 rpm for 10 min. Total RNA was isolated by the TRIzol reagent method (Gibco BRL), according to the user manual. RNA samples were treated with DNase (Invitrogen). RNA quality was monitored by spectrophotometry and electrophoresis. Total RNA was purified using Oligotex mRNA Kits (Qiagen) according to the manufacturer's directions and tested by spectrophotometry.

2.3. Suppression subtractive hybridization

This procedure was performed using the PCR-select™ cDNA Subtraction Kit (Clontech): cDNA was synthesized from 2 μg of mRNA from the T-TBT and T cells. The cDNA in which the specific transcripts are to be found is called the "tester cDNA", while the reference cDNA is called the "driver cDNA". Two subtractive libraries were made, one where the T-TBT cDNA was the tester and the T cDNA was the driver (forward-subtracted library) and another where these roles were reversed (T tester and T-TBT driver, reverse subtractive library). Both types of cDNA were digested by *Rsa*I and then extracted with phenol and chloroform. To make the tester cDNAs, 1 μl of each *Rsa*I digested cDNA was diluted with water and divided into two portions. One portion was ligated with adaptor 1 while the other portion was ligated with adaptor 2R. Each adaptor had a different PCR primer-annealing site. Two rounds of hybridization were then performed. In the preliminary hybridizations, an excess of driver cDNA was added to each sample of tester. The samples were heat denatured and allowed to anneal. Differentially expressed sequences were enriched in the single stranded molecules. In the second hybridization, samples from the two preliminary hybridizations were mixed together without denaturing, and freshly denatured driver cDNA was added to further enrich for differentially expressed sequences. The remaining single stranded tester cDNAs from each preliminary hybridization then annealed to form a new type of hybrids, double stranded tester molecules with different single stranded ends (from the two adaptors). Two rounds of PCR specifically amplify these differentially expressed sequences. Two subtracted cDNAs were obtained: reversed-subtracted cDNA (T was tester, and T-TBT was driver), and forward-subtracted cDNA (T-TBT was tester, and T was driver).

2.4. Cloning and dot blot hybridization

Second-step PCR products were directly cloned into the pGEM®-T easy vector (Promega), transformed into *E. coli* DH5α, and screened for blue/white colonies. For each subtracted