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Quantitative Analysis of Expression of Tributyltin (TBT)-Regulated Genes in TBT-Resistant *Pseudomonas aeruginosa* 25W

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Abstract—*Pseudomonas aeruginosa* 25W isolated from coastal seawater of Arabian Sea is highly resistant to tributyltin (TBT). We previously reported gene expression profile in this strain under the presence of 500 μM TBT, by DNA microarray analysis. In this study, to understand the mechanisms of TBT toxicity and resistance in *Pseudomonas aeruginosa* 25W, we quantified the expression of TBT respondent genes by real-time PCR. The results showed that two ribosomal protein genes (PA3600, PA3601), conserved hypothetical proteins, and cytochrome c550 gene (PA1983) were significantly down-regulated, which might have occurred by TBT toxicity. However, ribosomal protein gene (PA4242), ribosome-modulation factor gene (PA3049), cold-shock protein gene (PA1159) and elongation factor Tu gene (PA4265) were up-regulated. This suggests that the up-regulated genes in 25W strain might function to resist the toxicity of TBT.

Keywords: tributyltin resistance, *Pseudomonas aeruginosa*, real-time PCR

INTRODUCTION

Organotin compounds, including Tributyltin (TBT), have been used as a preservative for wood, antifouling agent in ship paints and on aquacultural nets. TBT is an organotin biocide which has toxicity against various organisms including eukaryotes and prokaryotes (Alzieu *et al*., 1989; von Ballmoos *et al*., 2004). Although use of TBT has been mostly prohibited by several countries, the effects on marine microbial community still continues by the residual TBT in the environment.

There have been a number of reports regarding TBT-resistant bacteria so far. However, only few reports on their resistance mechanisms have been published (Fukagawa and Suzuki, 1993; Jude *et al*., 2004). Recently, *Pseudomonas aeruginosa* 25W, which exhibits high resistance to TBT, was isolated from seawater of Arabian Sea (Upal *et al*., 2004). In preliminary transcriptome analysis using DNA microarray, gene expression changes in 25W strain were

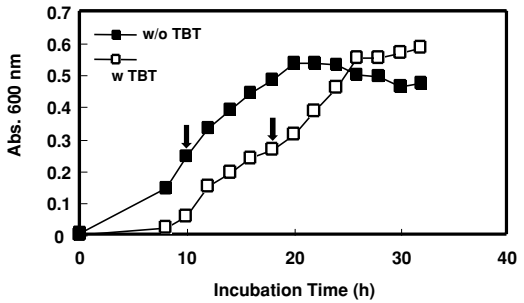

Fig. 1. Growth of *P. aeruginosa* 25W in the presence of 500 μM TBT. 25W strain was grown in M9 medium with 500 μM TBT (open square) or without TBT (closed square). The arrow indicates the time of cell-harvest.

observed in presence of TBT (Dubey *et al.*, 2006). Exposure to 500 μM TBT showed up- and down-regulations in some genes. In these regulated genes, we quantified the expression by real-time PCR to understand the TBT resistance mechanism of this resistant bacterium.

MATERIAL AND METHODS

Bacterial strain and culture condition

M9 medium supplemented with 0.2% glucose, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$ was used to grow *P. aeruginosa* 25W (Upal *et al.*, 2004). *P. aeruginosa* 25W was cultured in the absence and presence of 500 μM TBT at 37°C.

Total RNA extraction and cDNA synthesis

Cells were harvested at mid-log phase of the growth (Fig. 1). Total RNA was extracted from 10^9 cells using the RNeasy mini kit (QIAGEN) following the manufacturer's instructions and DNA was digested with RNase-Free DNase I (QIAGEN). The cDNA was synthesized with M-MLV Reverse Transcriptase (Invitrogen) and Random Primer (TaKaRa). RNase inhibitor (Amersham Biosciences) was added to the reaction mixture.

Quantitative real-time PCR

Relative quantification in real-time PCR was performed using SYBER Premix Ex Taq II (TaKaRa) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystem). The thermal cycling conditions were as follows: 95°C for 10 s and 40 cycles of 95°C for 5 s, 60°C for 31 s. Primers were designed using Primer3 Software. The cDNA was used as a template for PCR and each sample was assayed in duplicate. Threshold cycle values were used for calculation of fold change. 16S rRNA was used as internal control gene for normalization.

Table 1. List of target genes and result of quantification of the gene expression.

PA number*	Category	Encoded Gene	Fold change
PA1804	DNA replication, recombination, modification and repair	DNA-binding protein HU	–1.5
PA5348	DNA replication, recombination, modification and repair	Probable DNA-binding protein	1.34
PA0905	Translation, post-translational modification, degradation	Carbon storage regulator	–1.49
PA3049	Translation, post-translational modification, degradation	Ribosome modulation factor	1.67
PA3600	Translation, post-translational modification, degradation	Ribosomal protein L36	–50.76
PA3601	Translation, post-translational modification, degradation	50S ribosomal protein L31 type B	–49.75
PA4242	Translation, post-translational modification, degradation	50S ribosomal protein L36	1.67
PA4265	Translation, post-translational modification, degradation	Elongation factor Tu	1.50
PA1983	Energy metabolism; Carbon compound catabolism	Cytochrome c550	–18.31
PA2966	Fatty acid and phospholipid metabolism	Acyl carrier protein	1.18
PA4385	Chaperones and heat shock proteins	GroEL protein	–1.41
PA1159	Transcriptional regulators; Adaptation, Protection	Probable Acold-shock protein	1.62
PA0284	Hypothetical, unclassified, unknown	hypothetical protein	–2.00
PA5178	Hypothetical, unclassified, unknown	Conserved hypothetical protein	–3.53

*PA number corresponds to the genome annotation (http://www.pseudomonas.com).

RESULTS AND DISCUSSION

Although the 25W strain has high resistance to TBT, the growth of this strain was inhibited by the toxic effect of 500 μM TBT (Fig. 1). The toxicity of TBT on bacteria has not been well understood except for the inhibition of ATPase activity (von Ballmoos *et al*., 2004). Previous study indicated that transcriptional and translational genes are likely to be affected by exposure to 500 μM TBT (Dubey *et al*., 2006). We performed quantitative real-time PCR on genes which showed significant expression changes in DNA microarray analysis to confirm their expression levels in presence of 500 μM TBT.

As shown in Table 1, two ribosomal protein genes (PA3600, PA3601) and cytochrome c550 gene (PA1983) were significantly down-regulated. This suggests that the genes associated with translation and energy metabolism might be primarily affected. Meanwhile, TBT exposure up-regulated ribosomal protein gene (PA4242), ribosome-modulation factor gene (PA3049), elongation factor Tu gene (PA4265) and cold-shock protein gene (PA1159) (Table 1). This indicates that translation related genes showed up-regulation, suggesting that the up-regulation of these genes possibly protect the metabolism from TBT toxicity by increasing the translation machinery.

In this study, specific expression patterns were observed in five genes relating to translation processes. Among the up-regulated genes, PA4242 and PA4265 are located on the cluster (PA4237–4264) of ribosomal protein genes, whereas PA3049 is not located on this cluster. The cluster genes might be related to TBT resistance in this strain. On the other hand, down-regulated ribosomal protein genes can be controlled independently. We showed the effect of TBT on

gene expression of translational genes in this study. The toxic effect on the down-regulated genes might be compensated by up-regulation of some other genes to maintain protein synthesis system. The 25W strain will have some other resistance mechanisms than those detected in this study, because we have cloned various gene clusters responsible for TBT resistance, which contained membrane transport protein gene (data not shown). The new mechanism will be studied further.

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