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Microarray-Mediated Transcriptome Analysis of the Tributyltin (TBT)-Resistant Bacterium *Pseudomonas aeruginosa* 25W in the Presence of TBT

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The tributyltin (TBT)-resistant bacterium, *Pseudomonas aeruginosa* 25W, which was isolated in seawater from the Arabian Sea, was subjected to transcriptome analysis in the presence of high concentrations of TBT. Only slight effects were observed at TBT concentration of 50 μM, but exposure to 500 μM resulted in the upregulation of 6 genes and the downregulation of 75. Among the 75 downregulated genes, 53% (40 out of 75) were of hypothetical function, followed by 14 transcriptional regulation- and translation-associated genes. The results of this study indicated that although the 25W strain was highly resistant to TBT, high concentrations of TBT result in toxic effect on the transcriptional and translational levels. The target genes likely belong to a specific category of transcription- and translation-associated genes rather than to other gene categories.

***Keywords*:** Tributyltin resistance, *Pseudomonas aeruginosa*, microarray

Tributhyltin (TBT) is a toxic antifouling agent, which has been employed extensively in ship paint, and has also been identified as an immune system inhibitor and endocrine disruptor in humans. Thus, although the production, use and export of TBT have been prohibited in developed countries since the 1990s, some countries have continued to utilize this agent until the commencement of a complete prohibition, which scheduled to go into effect in 2008 (Rudel *et al*., 2003). Because of process by which TBT is degraded in marine environments is relevant to risk assessment and to estimations of the self-purifying capacity of those environments, trials targeted toward the isolation of TBT-degrading microbes have been underway for quite some time. TBT-resistant bacteria have previously been isolated from marine environments (Wuertz *et al*., 1991; Fukagawa *et al*., 1992; Suzuki *et al*., 1992), and some resistance genes (transport- and efflux-genes) have also been identified (Fukagawa and Suzuki, 1993; Jude *et al*., 2004). With regard to the bacterial degradation of TBT, Kawai *et al.* (1998) has reported finding TBT-degrading bacteria in river water, but no reports have yet been submitted asserting the detection of such bacteria in marine environments. Due to the limited nature of current knowledge regarding TBT resistance and degradation, we remain unable to elucidate precisely the mechanisms underlying TBT-resistance, much less TBT-degradation.

Recently, a *Pseudomonas aeruginosa* strain 25W, which is highly TBT-resistant, was isolated from surface waters of the Arabian Sea, off the shores of Goa, India (Roy *et al.,* 2004). *P. aeruginosa* has been reported to thrive not only in clinical and terrestrial environments, but also in marine environments (Kimata *et al*., 2004). The ecology and biochemical properties of marine *P. aeruginosa* have become a focus of interest for researchers in a variety of disciplines. For example, the International Genome Project has mapped the entire genome sequence of the *P. aeruginosa* strain PA01 (Stover *et al.,* 2000), which harbors in excess of 5,500 genes including many of hypothetical function. Palma's group has conducted a transcriptome analysis, using a DNA microarray, with the PA01 strain (Palma *et al*., 2003; Palma *et al*., 2004), which elucidated the responses of this strain to iron and oxidants. The DNA microarray procedure has undoubtedly proven is a powerful tool in investigations of this species.

The 25W strain appears to constitute a promising model with regard to TBT resistance in marine bacteria. Here, we describe our transcriptome analysis

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of the TBT-resistant 25W strain grown in the presence of high concentration of TBT.

Materials and Methods

Culture and RNA preparation

We grew the TBT-resistant *P. aeruginosa* 25W strain (Roy *et al*., 2004) in M9 medium (Miller, 1972), spiked with 50 or 500 μM TBT at 37°C, with shaking at 140 rpm. The *P. aeruginosa* PA01 strain was also used in this study, as a reference. Growth was monitored by recording absorbance at wavelength of 600 nm. Bacterial cells were harvested at mid-log phase. Total RNA was extracted from the cells with an ISOGEN extraction kit (Nippon Gene, Inc., Japan). Gene transcription is generally known to be affected by external stresses other than TBT. In this study, in order to preclude the influence of such effects, culture conditions and harvest timing in the TBT-treated and non-treated groups were carefully normalized. Furthermore, 2 concentration sets (50 and 500 μM) were employed in order to illustrate the differential gene responses to relatively lower and higher TBT concentrations.

Microarray assay

Although the PA01 DNA array is not completely identical to that of the 25W strain, the transcription products of the majority of the common genes could be evaluated by this method.

cDNA was prepared from RNA samples using Random Hexamer (Invitrogen, USA) and SuperScript II reverse transcriptase (Invitrogen, USA). The fragmented DNA was then employed in cDNA synthesis, via 3'-labeling with terminal deoxynucleotidyl transferase (Promega, USA) and Biotin-N6-ddATP (Perkin Elmer, USA). The labeled DNA fragments were then hybridized into a DNA microarray (NimbleGen Systems, USA) containing 5,567 genes of *P. aeruginosa* PA01 strain. The length of the oligo probes was 24-mer, and the number of perfect match probes was 17. The DNA microarrays were performed and hybridized at NimbleGen Systems Iceland LLC. Each of the gene spots was measured in duplicate. According to the fluorescent intensity of each of gene spots, Robust Multi-chip Analysis (RMA) algorithm analyses were conducted by the GeneFrontier Co. Japan. Genes evidencing statistically significant changes in expression were identified via t-tests ($p<0.05$).

Results and Discussion

The growth of the 25W strain is shown in Fig. 1, along with that of the PA01 strain. The 25W strain grew well in the presence of 50 and 500 μM TBT.

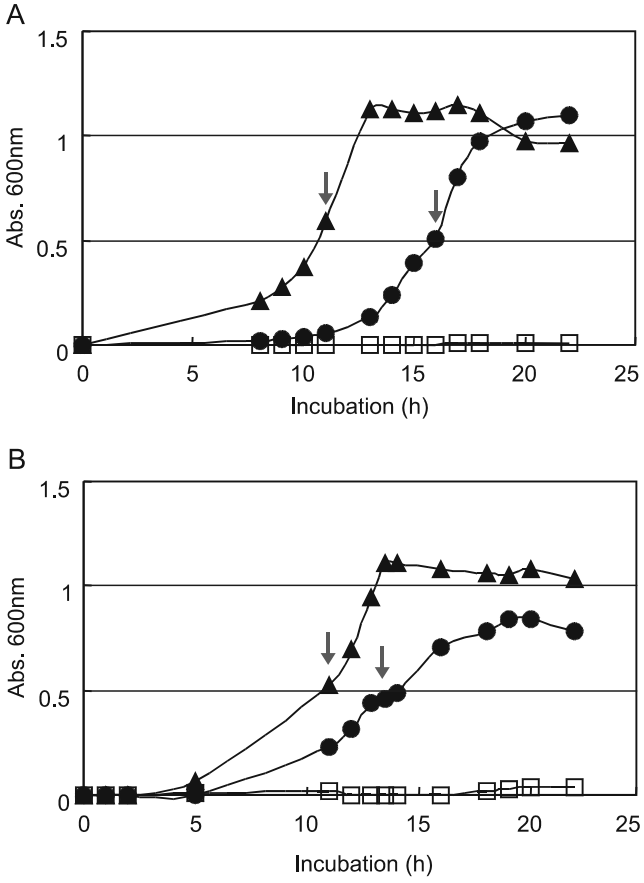

Fig. 1. Growth of *P. aeruginosa* 25W and PA01 in the presence of 50 μM TBT (A) and 500 μM TBT (B). Symbols are: closed triangle, 25W without TBT; closed circle, 25W with TBT; open square, PA01 with TBT. The arrow indicates time at which the cells were harvested.

The cell amount in the presence of 500 μM TBT was less than was observed in the 50 μM culture, which indicates that a high concentration of TBT suppressed the macromolecular synthesis. PA01 utterly failed to grow even in the 50 μM TBT culture, clearly indicating the susceptibility of this strain to TBT, whereas the 25W was confirmed to be highly resistant to TBT toxicity.

The 25W cells were harvested at mid-log growth phase in preparation for the DNA microarray assays. The up- and down-regulated genes are shown in Table 1, along with the fold-change values. Upon exposure to 50 μM TBT, only one gene (ribosomal protein) among the 5,567 genes in the DNA microarray was determined to have been upregulated, while another (hypothetical protein) was downregulated. This suggests that the SW25 strain is minimally susceptible to 50 μM TBT at the transcription level. The highest TBT concentration that has been reported in natural seawater is approximately 10^{-2} -10^{-3} mg/kg (equivalent to 50 nM) (Hoch, 2001). Marine bacteria are not

TABLE 1. List of genes up- and down-regulated by TBT

Up-regulated				
Gene ID[a]	Fold change	Gene product	TBT (μM)	PA numberb
881721	7.08	Probable DNA-binding protein	500	PA5348
882866	3.77	Ribosome modulation factor	500	PA3049
881721	2.18	Conserved hypothetical protein	500	PA5178
880387	1.90	Cytochrome c550	500	PA1983
880932	1.63	Hypothetical protein	500	PA1761
882179	1.47	Conserved hypothetical protein	500	PA0329
880142	1.10	Ribosomal protein L36	50	PA3600
Down-regulated				
Gene ID	Fold change	Gene product	TBT (μM)	PA number
879895	6.62	Acyl carrier protein	500	PA2966
879699	6.42	Hypothetical protein	500	PA0284
880137	4.32	50S ribosomal protein L31 type B	500	PA3601
878352	4.18	Carbon storage regulator	500	PA0905
877579	4.00	Probable cold-shock protein	500	PA1159
877870	3.47	Probable antioxidant protein	500	PA3450
881262	3.21	Outer membrane lipoprotein	500	PA1053
880142	3.01	Ribosomal protein L36	500	PA3600
881929	2.99	Putative sterol carrier protein	500	PA1830
881839	2.88	50S ribosomal protein L36	500	PA4242
881348	2.78	GroEL protein	500	PA4385
878308	2.76	DNA-binding protein HU	500	PA1804
882883	2.69	Hypothetical protein	500	PA2501
879531	2.61	Hypothetical protein	500	PA0201
881718	2.57	Elongation factor Tu	500	PA4265
881697	2.26	Elongation factor Tu	500	PA4277
881114	2.21	Hypothetical protein	500	PA4611
878015	2.20	Conserved hypothetical protein	500	PA4944
879441	2.10	Hypothetical protein	500	PA1414
881349	2.02	GroES protein	500	PA4386
882981	1.94	Lipoprotein, putative	500	PA3031
879431	1.87	Alkyl hydroperoxide reductase subunit C	500	PA0139
878026	1.85	Hypothetical protein	500	PA5446
879347	1.85	Probable cytochrome oxidase subunit (cbb3-type)	500	PA1557
879851	1.85	Outer membrane lipoprotein OprI precursor	500	PA2853
878396	1.84	Probable ATP-binding component of ABC transporter	500	PA0073
880423	1.77	Putative protein	500	PA4795
881823	1.66	S-adenosylmethionine decarboxylase proenzyme (EC 4.1.1.50)	500	PA4773
881702	1.65	50S ribosomal protein L10	500	PA4272
879463	1.58	Esterase/lipase/thioesterase family active site	500	PA1558
879774	1.57	Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding	500	PA3675
880294	1.52	SAM-dependent methyltransferases	500	PA0343
877672	1.52	5-carboxymethyl-2-hydroxymuconate isomerase	500	PA1966
881394	1.50	Probable cold-shock protein	500	PA0456
882957	1.49	Hypothetical protein	500	PA2464
881717	1.49	30S ribosomal protein S10	500	PA4264
879498	1.49	Cation/multidrug efflux pump	500	PA0359
882574	1.48	Protein yciI	500	PA3202
880339	1.48	50S ribosomal protein L19	500	PA3742
880548	1.48	RS21-C6-like protein (RS21-C6 protein)	500	PA4789
880462	1.47	Lipoprotein, putative	500	PA4791
878342	1.46	Nitrogen regulatory protein P-II 2	500	PA5288
879337	1.43	Hypothetical protein	500	PA1333
882474	1.43	Universal stress protein UspA and related nucleotide-binding proteins	500	PA3309
879375	1.40	Phosphoesterase, putative	500	PA0351
880269	1.39	Hypothetical protein	500	PA4801
878983	1.37	Conserved hypothetical protein	500	PA3431
879913	1.36	Ferredoxin-NADP+ reductase	500	PA3397
880209	1.36	Peptidyl-prolyl cis-trans isomerase C2	500	PA4176
881761	1.31	30S ribosomal protein S3	500	PA4257
879307	1.29	Formamidopyrimidine-DNA glycosylase	500	PA0357
881108	1.28	Putative copper export protein	500	PA4610
882186	1.28	Hypothetical protein	500	PA0526
881562	1.24	MoxR-like ATPases	500	PA4322
881771	1.24	50S ribosomal protein L29	500	PA4255
881623	1.17	Sulfate-binding protein of ABC transporter	500	PA1493
877837	1.15	Probable ATP-dependent protease	500	PA0779
882940	1.06	Hypothetical protein	500	PA2459
879902	1.05	Reverse transcriptase (EC 2.7.7.49)	500	PA0715
878475	1.05	Hypothetical protein	500	PA2037
881116	1.05	Hypothetical protein	500	PA1371
882297	1.05	Probable transcriptional regulator	500	PA2227
882913	1.05	Serine/threonine protein kinase	500	PA2461
882581	1.05	LPS biosynthesis protein WbpG	500	PA3150
881109	1.05	Superfamily I DNA and RNA helicases	500	PA1372
882652	1.05	B-band O-antigen polymerase	500	PA3154
880072	1.05	Hypothetical protein	500	PA0642
879819	1.05	Pyocin S2 immunity protein	500	PA1151
878059	1.05	Hypothetical protein	500	PA1152
878006	1.04	Hypothetical protein	500	PA2226
879565	1.04	Hypothetical protein	500	PA1369
878612	1.04	Hypothetical protein	500	PA0981
879713	1.04	Hypothetical protein	500	PA2224
879512	1.04	6-aminohexanoate-dimer hydrolase (EC 3.5.1.46)	500	PA2228
879580	1.03	Hypothetical protein	500	PA2222
881114	1.89	Hypothetical protein	50	PA4611

[a]GeneID can be accessed in NCBI database. [b]PA number can be accesssed in www.pseudomonas.com.

routinely exposed to such high TBT concentrations (50 μM), which would suggest that the selection pressure in seawater is not particularly profound. However, we did detect highly-resistant bacteria in seawater, such as 25W (this study) which is resistant to 2 mM TBT, as well as *Alteromonas* sp. M-1, which is resistant to 125 μM TBT (Fukagawa *et al*., 1992; Roy *et al*., 2004). These findings indicate that natural marine bacterial communities harbor spontaneously TBT-resistant organisms. Recently, Jude *et al*. (2004) reported association of a multidrug efflux pump cluster gene, TbtABM, with TBT resistance in *P. stutzeri*. In this study, no such efflux encoding genes were upregulated in the 25W cultures, thereby indicating that the production of proteins such as TbtABM is not accelerated by TBT-associated stress.

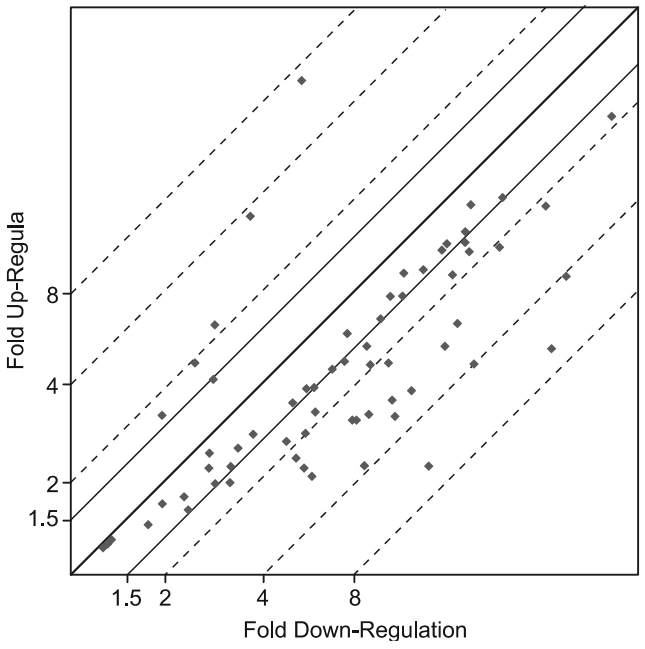

Fig. 2. Scatter plot of the DNA microarray transcriptome assay of cells grown in the presence of 500 μM TBT.

We then cultured the 25W strain in the presence of a higher concentration (500 μM) of TBT. As can be seen in Fig. 2, the scatter plot indicates statistically significant changes occurring in 81 genes, with 6 upregulated and 75 downregulated genes (Table 1). Three genes evidenced upregulation in excess of 2-fold, and these genes encoded for a probable DNA-binding protein, a ribosome modulation factor, and a conserved hypothetical protein. These genes are believed to contribute to the resistance observed in this strain. Such upregulated genes may be inducible genes that, under TBT-associated stress, function to protect or accelerate protein synthesis.

Among the 75 downregulated genes, 20 evidenced expressional changes of greater than 2-fold (box in Table 1). The downregulation of these genes may well be induced by TBT toxicity, which suggests that high TBT concentrations would generate stresses that result in an inhibition of transcription in these genes. The set of 20 highly downregulated genes contained 5 translation-related genes, including translation, post-translation, and degradation-associated genes. All of

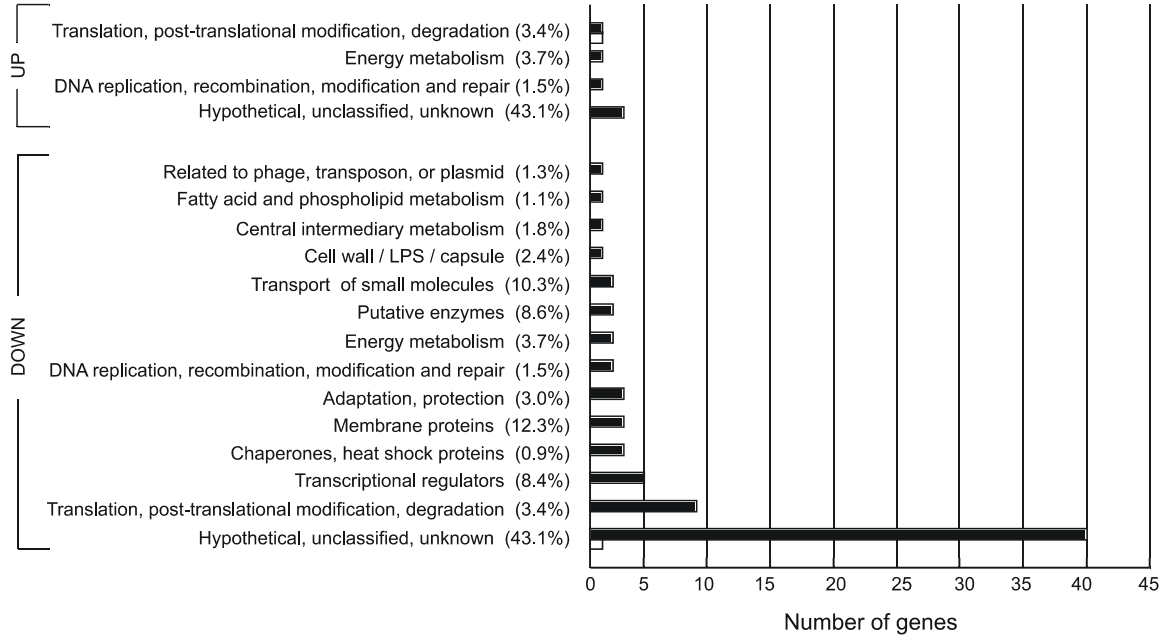

Fig. 3. Functional classification of genes differentially regulated via exposure to TBT. Open bar, under 50 μM TBT; closed bar, under 500 μM TBT. Numbers in parentheses indicate percentage occupied by each category out of a total of 5,567 genes.

the differentially regulated genes were categorized, as is shown in Fig. 3. The percentage of each category, in relation to the total of 5,567 genes, is also shown (in parentheses) in this figure. A total of 9 translation-related genes were downregulated. One hundred ninety genes were categorized in the translation-related category of *P. aeruginosa* (http://www.pseudomonas.com/index.html), corresponding to 3.4% of the 5,567 total genes. The 9 downregulated translation-associated genes constituted 12.0% of the 75 total downregulated genes. This finding may point to a specific target of TBT in the transcription step. Furthermore, if one includes the 5 transcriptional regulator genes among the downregulated genes, a total of 14 of the total downregulated genes can be safely associated with transcription and translation steps (18.7%). Members of other gene categories, *e.g.* membrane proteins, accounted for 12.3% of the total genes, comprising the maximum number among all categories. However, downregulated membrane proteins comprised only 4.0% of the total of 75 downregulated genes, which corroborates the conclusion that translation-associated genes are affected by TBT more profoundly than any other category of genes. Membrane-related proteins have been associated not only with the mechanism underlying TBT-resistance (Fukagawa and Suzuki, 1992; Jude *et al*., 2004), but also with inhibition targets (von Ballmoos *et al*., 2004). However, the results of our study indicated neither an acceleration nor an inhibition of membrane proteins occurring in the 25W strain.

Most TBT toxicity studies have been conducted at the protein level, including a recent advanced study concerning the ion channel inhibition of F-ATP synthase (Hoch, 2001). Our study revealed the toxic effects of TBT at the transcriptome level, which, to the best of our knowledge, is the first such evidence thus far reported. We suggest that TBT toxicity may manifest in the transcription step only for specific genes, rather than in all gene categories.

The majority of downregulated genes characterized in this study were of either hypothetical function or as-yet unclassified (53.0%), as can be seen in Fig. 3. This may suggest the presence of further, as-yet unknown, specific target genes for inhibition at the transcription level. However, as the category of hypothetical proteins occupied such a large portion (43.1%) of the 5,567 total genes, we remain unable to clarify this, at least until further studies are conducted regarding the TBT-mediated regulation of specific genes.

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