

## ***Aeromonas molluscorum* Av27: A Potential Natural Tool for TBT Decontamination**

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**Abstract**—Tributyltin (TBT) is a toxic compound employed in several industrial processes. Its discharge into the environment has been recognized worldwide as a pollution problem. The importance of bacteria in decontamination processes has been long acknowledged. The aim of this work was to screen TBT resistant/degrading bacteria in order to investigate the involved mechanisms. *Aeromonas molluscorum* Av27, isolated from Ria de Aveiro (Portugal), showed high TBT resistance (up to 3 mM). From a genomic library, a TBT resistant clone (5.4 kbp fragment) was selected. In this fragment, ORF P6 was able to confer TBT resistance to *E. coli*. P6 has homology with a *sugE* gene, which encodes SugE protein, belonging to small multidrug resistant family, a lipophilic drug transporter. Reverse transcriptase-PCR analysis indicated that increased expression of the *sugE* gene was found in the original strain (*A. molluscorum* Av27) when the cells were grown in the presence of high concentrations of TBT. *A. molluscorum* Av27 degrades TBT in dibutyltin (DBT) and monobutyltin (MBT) and uses it as carbon source. Microcosm experiments with TBT contaminated sediments revealed that the addition of Av27 strain does not affect the natural bacterial community. Given the interesting characteristics exhibited by Av27, it can potentially be used to develop a bioreporter system to monitor on-site TBT contamination and also be employed as a mediator of TBT polluted sites.

**Keywords:** TBT, resistance, *Aeromonas molluscorum*, *sugE*, gene expression, DGGE

### INTRODUCTION

Tributyltin (TBT) is an organotin compound broadly employed as fungicide, bactericide, pesticide, wood preservative, PVC stabilizer and a component of antifouling paints. Although the production, use and export of TBT have been prohibited in developed countries since the 1990s by the International Maritime Organization (IMO), some countries are still using it (Rudel, 2003). European

Union (EU) followed the rational of a convention and introduced the Directive 2002/62/EC that bans the application of organotin antifouling paints on EU boats after 1 January 2003 and forbids its usage by any boats after 2008. In estuarine waters the typical half-life of TBT is 6 to 7 d at 28°C; however, in deeper anoxic sediments degradation is much slower (1.9 to 3.8 yr), remaining as a reservoir and source of TBT for many years. Consequently, TBT pollution has been recognized as a serious problem around the world.

Organotins can be highly toxic to many eukaryotic and prokaryotic organisms and have also been identified as immune system inhibitors and endocrine disruptors in humans (Gibbs and Bryan, 1996; Dubey and Roy, 2003; Dubey *et al.*, 2006). In many aquatic organisms, its toxic effect is also recognized; the concentration of 1–2 ng l<sup>-1</sup> (nanomolar level) causes growth suppression, immune suppression, and imposex in higher animals (White *et al.*, 1999; Hoch, 2001). While inorganic forms of tin are of relatively low toxicity, the more lipid-soluble organotins can be highly toxic to bacteria and fungi (Dubey and Roy, 2003). Generally, trisubstituted organotins (TBT) are more toxic than di-(DBT) and monosubstituted (MBT) compounds, and seem to exert toxicity through their interaction with membrane lipids. Organotin compounds are toxic to both Gram-positive and Gram-negative bacteria isolated from sediment; nevertheless, the former showed increased sensitivity to triorganotins (Mendo *et al.*, 2003; Cruz *et al.*, 2007).

Several mechanisms have been proposed that could be involved in TBT resistance in bacteria: (i) transformation into less toxic compounds (DBT and MBT) by abiotic and biotic mechanisms (Clark *et al.*, 1988; Dowson *et al.*, 1996; Pain and Cooney, 1998); (ii) exclusion of the compound from the cell mediated by a multidrug efflux pump (Jude *et al.*, 2004); (iii) degradation/metabolic utilization as a carbon source (Kawai *et al.*, 1998); (iv) bioaccumulation into the cell without breakdown of the compound (Fukagawa *et al.*, 1994).

*Aeromonas molluscorum* Av27 was isolated from an area moderately contaminated by TBT, in Ria de Aveiro, Portugal (Cruz *et al.*, 2007). It is highly resistant to TBT (up to 3 mM) and possesses the ability to degrade it into DBT and MBT (less toxic compounds), and can use TBT as carbon source (Cruz *et al.*, 2007), thus suggesting the potential application in natural remediation of TBT contaminated areas. Given the role played by bacteria in biogeochemical cycles, the catabolic activities of introduced microorganisms are important in bioremediation technologies for the restoration of polluted environments (Heinaru *et al.*, 2005).

The main aim of the present work was to identify the gene(s) involved in TBT resistance in *A. molluscorum* Av27 that could be used in the future to develop a biosensor system to detect TBT from the environment. Microcosms experiments were performed to investigate the presence of Av27 in the natural environment and its impact and influence on resident bacterial community structure.

## MATERIALS AND METHODS

### *Bacterial strains, identification and growth conditions*

*Aeromonas molluscorum* Av27 (previously identified as *Aeromonas veronni* biovar *sobria* (Cruz *et al.*, 2007)) was isolated from sediments from Ria de Aveiro, Portugal. Av27 strain was selected to study the gene(s) that are involved in TBT resistance based on their TBT resistance profile up to 3 mM (Cruz *et al.*, 2007). Cloning and sub cloning experiments were performed in *E. coli* HB101 competent cells (Promega). Bacterial strains were grown with shaking (200 strokes  $\text{min}^{-1}$ ) at 26°C (*A. molluscorum* Av27) or 37°C (*E. coli*) in TSB medium (Merck) or in LB broth (Merck), respectively. Selecting agents for plasmids were added to growth media when appropriate: ampicillin (*E. coli*) at 50  $\mu\text{g ml}^{-1}$  and TBTC1 (Fluka) from 0.05 mM to 3 mM of concentration.

### *DNA extraction and analysis*

Total DNA was extracted using “Genomic DNA purification kit” (MBI Fermentas). GFX “Micro Plasmid Prep kit” (Amersham Biosciences) was used to perform midi-preparations of plasmid DNA and mini-preparations were achieved by alkaline lyses procedure (Sambrook *et al.*, 1989). Restriction digests were carried out according to the supplier’s instructions (MBI Fermentas) and analyzed by electrophoresis on agarose gels in TAE (Sambrook *et al.*, 1989).

### *Cloning experiment*

*Bam*HI genomic DNA from *A. molluscorum* (2  $\mu\text{g}$ ) were ligated (T4 DNA ligase, MBI Fermentas) to 1  $\mu\text{g}$  of *Bam*HI digested-pUC19, treated with alkaline phosphatase (MBI Fermentas), and transformed into *E. coli* HB101 cells according to the manufacturer’s instructions (Promega). Screening for TBT-resistant clones was carried out primarily on LB agar plates with ampicillin. Afterwards, ampicillin-resistant clones were transferred to LB agar plates containing ampicillin and 2 mM of TBTC1. From all the clones, one TBT-resistant clone 69, containing an insert with 5.4 kbp, was selected for further studies.

### *DNA sequencing and analysis*

Nucleotide sequence of the fragment inserted into pUC19 was determined in an ABI PRISM 3700 (MacroGen Company: <http://www.macrogen.com>). Nucleotide and deduced amino acid sequences were analysed using GENETYX-WIN version 5.1.1 and the BLAST program available from National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/BLAST/>). In clone 69, six different ORF’s were identified as candidate genes involved in resistance TBT.

### *Sub cloning experiments*

Specific primers were designed to amplify the candidate ORF’s that were sub cloned in HB101 cells (see Table 1). Designed primers contained at each

Table 1. Oligonucleotides used to amplify each ORF from clone 69.

ORF to amplify	Oligonucleotide name	Oligonucleotide sequence (5'-3')
P1	69A	GAGAGAGAA TTC ATG ATG CTG CAC CTT AAG CTC GGC
	69B	GAGAGAAAG CTT TCA GCG TCG TGA CCA GCA CCC
P2	69C	GAGAGAGAA TTC ATG ACC ATA ACC TCT GAG CAC GGT
	69D	GAGAGAAAG CTT TCA ACT TTC GAT CAA CTC GTG ACG
P3	69E	GAGAGAGAA TTC ATG AGT AGC CAG GCG CCA GCC
	69F	GAGAGAAAG CTT CTA TGA CTG TCC CTT GCC GAG CCT
P4	69G	GAGAGAGAA TTC ATG GCG GAG GAT CTC ACC GAG TAT
	69H	GAGAGAAAG CTT TCA GAG GTT ATG GTC ATA CTG CAG
P5	69I	GAGAGAGAA TTC ATG ACA GAC CAC ACT TTT ATA CCC
	69J	GAGAGAAAG CTT TTA CTG CCA GTT GAA GTT GCG TTT
P6	69K	GAGAGAGAA TTC ATG TTC ATG CCC TGG ATA TTG CTG
	69L	GAGAGAAAG CTT TCA ACC GAT GGC TTT GAG ACC CAG

terminal the *Bam*HI and *Hind*III restriction sites (Table 1), allowing ligation into the *Bam*HI/*Hind*III pUC19 vector. Ligated fragments were transformed in *E. coli* HB101 competent cells.

PCR was carried out in 50  $\mu$ l reaction mixture consisting of 3.0 mM MgCl<sub>2</sub>, 0.3 pmol of each oligonucleotide (Table 1), 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 1  $\times$  green GoTaq<sup>®</sup>Flexi Buffer (Promega, USA), 1 U GoTaq<sup>®</sup> DNA polymerase (Promega, USA), 10–100 ng plasmid DNA of clone 69. Twenty-five amplification cycles were carried out under the following conditions: denaturation at 95°C for 0.5 min, annealing at 50–62°C for 1 min, and extension at 72°C for 1 min kb<sup>-1</sup>. Growth of each sub clone, to check for TBT resistance, was carried out in LB medium with ampicillin, containing 100  $\mu$ M of TBT, at 250 rpm, 37°C. Growth was recorded as a change of optical density at Abs<sub>600nm</sub>. A negative control consisted of pUC19 vector inserted into competent cells (clone pUC19). Clone 69 acted as the positive control.

Sub clone P6, showed a growth similar to that of the positive control (clone 69) and was used for further studies.

#### *Reverse transcription (RT) PCR experiments*

RNA was prepared from cultures of: (i) *A. molluscorum*; (ii) sub clone P6 (containing *sugE*-like gene) and (iii) sub clone pUC19 (containing only the pUC19 vector). Optical density of each culture was at Abs<sub>600nm</sub> = 1. RNA was extracted with “NucleoSpin RNA II” that includes a DNase treatment, according to the supplier’s instructions (Macherey-Nagel, Germany). cDNA was synthesized using random primers from “First Strand cDNA Synthesis kit” (Fermentas, USA). The RT-PCR analysis was undertaken with and without RT enzyme (as a control). PCR was then prepared using oligonucleotides 69K and 69L (shown in Table 1) that amplified ORF P6.

Table 2. Conditions employed for the microcosm experiment.

Condition	Description
I	Sediment + 50 $\mu\text{M}$ TBT + Av27
II	Sediment + 50 $\mu\text{M}$ TBT
III	Sediment + ethanol

### *Microbial community analyses: PCR-DGGE analysis of bacterial 16S rDNA gene*

A microcosm experiment was performed in three different conditions (Table 2). Sediment and water were collected from Ria de Aveiro and kept at 4°C until used. pH, salinity and temperature of the water and sediment collected were recorded.

The microcosm conditions were set into glass flasks and were prepared as described in Table 2. Each flask contained: 220 g of sediment, 200 ml of water and 50  $\mu\text{M}$  of TBT; each experimental condition was prepared in triplicate. The microcosm contained non-filtered seawater and sediment constituted with fine clay, millimeter size sandy and muddy materials.

Microcosm samples were incubated at room temperature, in the dark. During the 30 days of incubation, the microcosm's samples were mixed to simulate real environment. After 0 and 30 days, subsamples of approximately 2 g were withdrawn from the flasks to DGGE analysis. After being frozen in liquid nitrogen, samples were kept at -70°C until analysis. From each sample, total DNA was extracted using "Ultraclean Soil DNA Isolation kit" (MoBio laboratories, Inc.). DNA was used to amplify 16S rDNA gene. PCR was carried out in 50  $\mu\text{l}$  reaction mixture consisting of 3.0 mM  $\text{MgCl}_2$ , 0.3 pmol of each oligonucleotide (518R: 5'-ATTACCGCGGCTGCTGG-3' and 338F: 5'-GCCGCCCGCCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3'), 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 5% DMSO, 1  $\times$   $\text{Mg}^{2+}$ -free DyNAzyme™ buffer (Finnzymes, Finland), 1 U DyNAzyme II DNA Polymerase (Finnzymes, Finland), 10–100 ng total DNA. Thirty amplification cycles were carried out under the following conditions: initial denaturation at 94°C for 5 min, denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, extension at 72°C for 0.5 min, and final extension 72°C for 30 min.

PCR products of bacterial 16S rDNA gene were analyzed on a 1% agarose gel. 50 ng of each PCR product was loaded in each gel lane and were separated for 15 min at 20 V for 16 h at 75 V, on 37–65% denaturing gradient gels. After electrophoresis gels were stained with ethidium bromide, and digitalized. The number of bands and their position on the DGGE gel were analyzed.

### *Accession number*

DNA sequences of the P6 sub clone—*sugE*-like—identified in this study is

deposited in the GenBank™ sequence database under the accession number FJ225136.

## RESULTS AND DISCUSSION

A *Bam*HI DNA library of *A. molluscorum* Av27 was constructed in *E. coli* HB101. 14 clones were found to be resistant to 3 mM of TBT. One of those clones (clone 69) contained a fragment of ~5.4 kbp. The nucleotide sequence of the fragment was determined and compared with other sequences deposited in the GenBank database. Six different ORFs were defined in clone 69, constituting the candidate genes responsible for TBT resistance (Table 3).

Each of the identified ORF's were individually sub cloned into competent *E. coli* HB101 cells, and growth experiments in liquid media were performed with each of the sub clone in LB medium containing ampicillin and 100  $\mu$ M TBT. Only sub clones P2, P3, P4 and P6 of clone 69, were resistant to TBT (Fig. 1). Growth was compared to that of the clone 69 and also to the negative control (pUC19).

ORF P6 showed the highest resistance to TBT and was therefore selected to further studies. It contains a fragment of 315 bp, with high homology with *sugE* gene that encodes the SugE protein (11.88 kDa) that is involved in the transport of lipophilic drugs (Sikora and Turner, 2005) and belongs to “small multidrug resistant proteins”. It was reported that SugE expression confer a highly specific drug resistance phenotype to quaternary ammonium compounds (Chung and Saier, 2002). TBT and its degradation products (DBT and MBT) are lipophilic compounds and it is possible that the affinity of this compounds to the SugE-like protein, present in the inner membrane, may play a role in the transport of TBT, DBT or MBT outside or inside the bacterial cell, this mechanism is not very clear yet.

### *Analysis of transcription levels by reverse transcription (RT)-PCR*

Analysis of expression levels of *sugE*-like gene by RT-PCR (Fig. 2) showed the presence of a transcript of 315 bp fragment in the TBT-resistant strain, Av27. An increased expression level of this gene was observed as the TBT concentration in the culture media increased. No apparent differences were detected on the expression levels of *sugE* clone (*sugE* clone), suggesting that in this clone this gene is constitutively expressed. As expected, in clone pUC19 (plasmid vector with no insert), no amplification was observed. Two different negative controls were prepared: (i) without cDNA; and (ii) without reverse transcriptase enzyme.

RT-PCR analysis was used as a qualitative method to detect the gene expression level of *sugE*-like gene. Results showed an increased expression of this gene, as revealed by an increase on the mRNA level when cells are grown in the presence of increasingly higher TBT concentrations.

### *Microbial community analyses: PCR-DGGE analysis of bacterial 16S rDNA gene*

Bearing in mind that *sugE*-like gene can be used to develop a biosensor

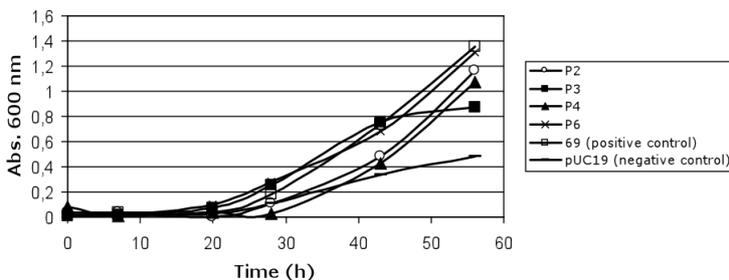


Fig. 1. Growth of clone 69 (positive control: resistant to TBT), pUC19 (negative control: *E. coli* HB101 containing only pUC19) and TBT resistant sub clones, containing individual ORFs of clone 69, in LB medium containing ampicillin and 100  $\mu$ M TBT. Growth occurred at 37°C for 56 h and 250 rpm, and it was monitored as a change of absorbance at 600 nm.

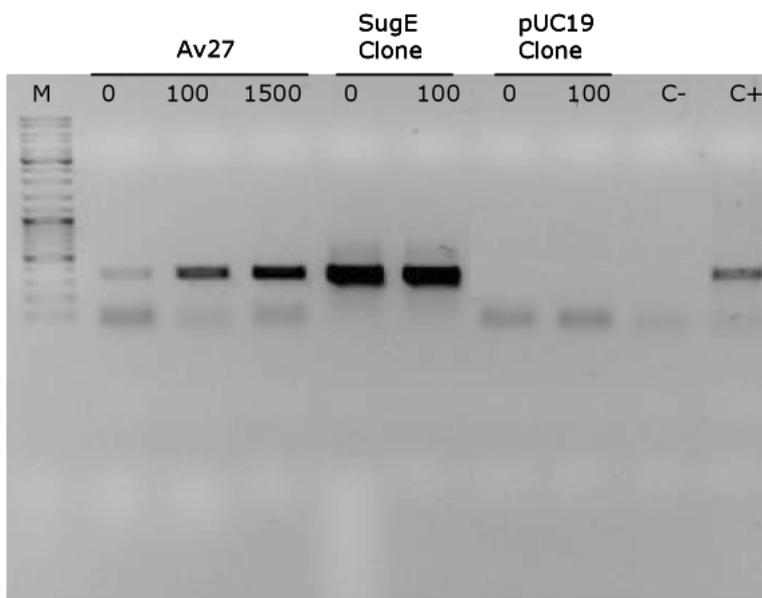


Fig. 2. RT-PCR analysis of TBT sensitive and resistant strain/clones. M, GeneRuler DNA ladder mix (Fermentas); C-, prepared without cDNA; C+, prepared with tDNA of Av27 strain.

system to detect TBT in contaminated areas, it is necessary to evaluate the impact of the introduction of Av27 in the natural environment. With that purpose, microcosms experiments should be performed with the attempt of simulating the environment conditions in the laboratory.

The values of pH, salinity and temperature of the water and sediment

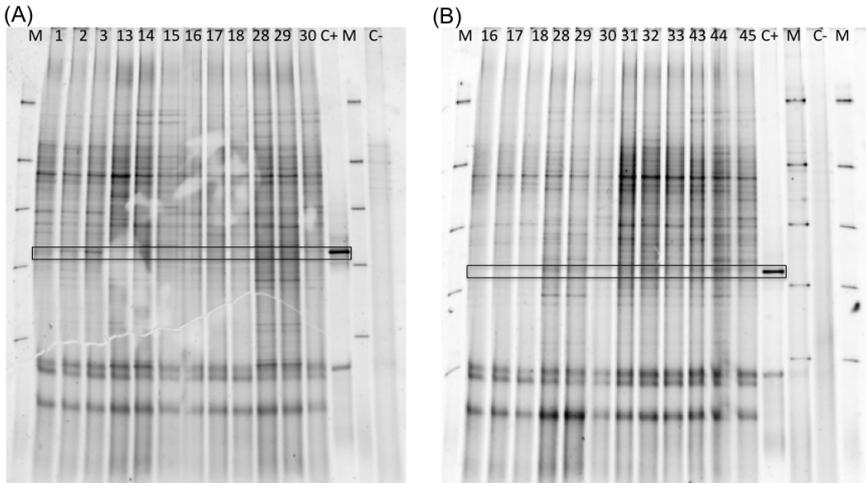


Fig. 3. DGGE analysis of V3 region of rDNA 16S of microcosm samples. The correspondence of each sample is shown in table 4. C-, PCR performed without DNA; C+, PCR performed using total DNA of Av27. Lane M: DGGE marker I (Wako, Japan).

collected in Ria de Aveiro, to perform the microcosm experiment, were 7.5, 35  $\text{g}\cdot\text{l}^{-1}$  and 26°C, respectively.

A preliminary experiment was performed to evaluate the influence of Av27 and TBT in the natural microbial community. Figure 3(A) shows that the samples containing TBT and inoculated with the resistant strain Av27 (1, 2, 3, 13, 14, 15) and without the Av27 strain (16, 17, 18, 28, 29, 39) at times 0 and 30. In Fig. 3(B), are shown the DGGE profiles for the samples with (16, 17, 18, 28, 29, 39) and without (31, 32, 33, 43, 44, 45) TBT at times 0 and 30 (Table 4). It is possible to observe that the microbial community is relatively stable along the 30 days. As expected, a higher number of bands appeared, corresponding to new groups of bacteria, after 30 days, resulting from the incubation time. The addition of the resistant strain does not seem to influence the community. After 30 days, the dominance of Av27 is not relevant (see rectangle). Also, the addition of TBT does not seem to affect the bacterial community significantly. However further experiments are needed where organotin analysis of the sediments have to be performed to evaluate the ability of the natural resident community plus Av27 strain to degrade TBT. The results suggest that the natural microbial community is already adapted to the presence of TBT in the natural environment. The addition of Av27 does not affect the natural community. Therefore, it is clear that the Av27 strain can be potentially employed in future bioremediation studies.

The results shown in this study are very promising and open new perspectives for the biotechnological applications of *A. molluscorum* Av27 for the development of a biosensor system to detect TBT in the environment and ultimately in the bioremediation of TBT contaminated areas.

Table 3. Homologies and percentage of identity of the ORFs identified in clone 69.

ORF	% identity with	Protein homology
P1	95%; <i>A. hydrophila</i>	Excinuclease ABC subunit B
P2	85%; <i>A. salmonicida</i>	Excinuclease ABC subunit B
P3	71%; <i>A. hydrophila</i>	Ded A
P4	91%; <i>A. hydrophila</i>	Excinuclease ABC subunit B
P5	89%; <i>A. salmonicida</i>	Hypothetical protein ASA-2350
P6	84%; <i>A. hydrophila</i>	SugE

Table 4. Samples used in microcosm experiment performed during 30 days.

Condition	Description	Time 0 d	Time 30 d
I	Sed + TBT + Av27	1, 2, 3	13, 14, 15
II	Sed + TBT	16, 17, 18	28, 29, 30
III	Sed + ethanol	31, 32, 33	43, 44, 45

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