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***Aeromonas veronii*, a tributyltin (TBT) degrading bacterium isolated
from an estuarine environment, Ria de Aveiro in Portugal**

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ABSTRACT

Organotin compounds are used in a variety of industrial processes therefore their subsequent discharge into the environment is widespread.

Bacteria play an important role in biogeochemical transformations acting as natural decontamination agents. Therefore, screening for tributyltin (TBT)-resistant and -degrading bacteria is relevant for the selection of isolates with decontamination ability of these polluted areas. With this purpose, fifty strains were isolated from sediment and water from Ria de Aveiro and their tolerance to TBT, up to 3 mM, was evaluated. Generally, occurrence of highly TBT-resistant bacteria was observed, and Gram negative bacteria exhibited more tolerance to TBT than Gram positive bacteria. A memory response was observed when bacteria were progressively exposed to increasingly higher TBT concentrations. One isolate, *Aeromonas veronii* Av27, highly resistant to TBT (3 mM) uses this compound as carbon source and degrades it to less toxic compounds.

1. INTRODUCTION

Tributyltin (TBT) is an organotin compound broadly applied as fungicide, bactericide, pesticide, wood preservative, PVC stabilizer and 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 have continued to utilize this agent until its complete prohibition, which was scheduled to go into effect in 2008 (Rudel et al., 2003). Consequently, TBT pollution has been recognized as a serious problem around the world.

Ria de Aveiro is an important estuarine system on the north-west coast of Portugal. It is a shallow system that occupies an area of 43 km² at low tide and its bottom consists mainly of muddy sediments. The most important sources of organotin pollution are the port, dockyards and marinas, where TBT levels range from 9-42 ng TBT-Sn l⁻¹ in the water and up to 88 ng TBT-Sn g⁻¹ dry weight total sediment (Barroso et al., 2000).

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, Tokashiki and Suzuki, 2006). In many aquatic organisms, its toxic effect is also recognized; the concentration of 1–2 ng l⁻¹ (nanomolar level) causes growth suppression, immunosuppression, and imposex in higher animals (White et al., 1999; Hoch, 2001).

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. It has been reported that organotin compounds are toxic to both Gram positive and Gram negative bacteria collected from sediment; nevertheless, the former showed increased sensitivity to triorganotins (Mendo et al., 2003).

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).

The catabolic activities of introduced microorganisms play an important role in bioremediation technologies for the restoration of polluted environments (Heinaru et al., 2005). In this context, a given bacterial population might perform the reverse reaction on tin compounds by demethylation or methylation of TBT into less toxic derivatives (Jude et al., 2004). Given the function played by bacteria in biogeochemical cycles, the identification and characterization of TBT pollutant-degrading bacterial strains is crucial. Knowledge of their physiology and genetics is fundamental for their future application as natural decontamination agents.

In the present study we aimed to isolate and identify bacteria resistant to TBT that could be used and developed as biosensors for TBT pollution. The ability to degrade TBT into DBT and MBT (less toxic compounds), and its use as carbon source, was evaluated on selected bacteria. One particular isolate, *A. veronii* Av27, fulfilled all the characteristics needed for its future application as a TBT sensor and also for the remediation of TBT polluted areas.

2. MATERIALS AND METHODS

2.1. Bacterial isolation and identification

Bacterial strains were isolated from sediment (November 2001) (Mendo et al., 2003) and water (Janeiro 2004) from Ria de Aveiro, a shallow estuarine system, near Aveiro (40°38'N, 8°41'W), in the north-west coast of Portugal. The TBT concentrations recorded were 39 ng Sn g⁻¹ and 79 ng Sn l⁻¹ in sediment and water, respectively (Barroso et al., 2000).

Sediment cores (10 cm in diameter and 5 cm depth) were used to collect the sediment that was extruded into sterile flasks and processed immediately. Bacteria were isolated by the following steps: 10 g of sediment were mixed with 100 ml of sterile artificial seawater, for 30 min; serial dilutions, up to 10⁻¹⁰, were plated on Tryptic Soy Agar (TSA) (Merck, Germany), Plate Count Agar (PCA) (Merck, Germany) and Marine Broth Agar (MBA) (Difco, USA); Petri dishes were incubated overnight at 20 °C.

Water samples were collected from overlaying water (20 cm) using an immersed sterile bottle during the flood tide. Samples were transported to the laboratory and processed within 4 h.

Bacteria were isolated by following steps: serial dilutions up to 10⁻⁶ in sterile artificial seawater were spread in MBA, TSA and PCA; plates were incubated overnight at 20 °C.

Morphologically different colonies were picked from the plates and re-streaked on the same medium to ensure purity. Fifty isolates were selected and identified using Biolog Microlog 3 4.01 C, Vitek[®] and API[®] systems (bioMérieux, Hazelwood, MO, USA). Whenever necessary, 16S rDNA sequence was determined after PCR amplification of the corresponding amplicon (Muyzer et al., 1993). Nucleotide amino acid sequences were analysed using BioEdit Sequence Alignment Editor (Isis Pharmaceuticals) and the BLAST program available from the National Institute for Biotechnology Information server (<http://www.blast.genome.ad.jp>). Strains were maintained in

TSA and MBA plates at 4 °C and were transferred monthly to fresh medium. Long-term storage was performed in 45 % glycerol at -70 °C.

2.2. TBT sensitivity

Gradient plates were prepared with TBTCI (97 %) (Fluka, Switzerland) incorporated in TSA medium, with concentrations ranging from 0 to 3 mM (subsequently the compound will simply be referred to as TBT). TBT stock solution was prepared in ethanol and stored in the dark at 4 °C. Each plate, with the dimensions of 11x11 cm, consisted of a first layer of TSA (50 ml) containing 3 mM of TBT that was left to stand with a slight inclination; after the agar has solidified, 50 ml of TSA were layered on the top of the first layer (adapted from Metzenberg and Grotelueschen, 1992). Bacteria to be tested were grown on TSB at 250 rpm (Sanyo incubator shaker) until they reached an optical density ($OD_{600\text{ nm}}$) of 1 (approximately after 10 h of incubation) at 26 °C. A temperature of 26 °C was chosen, as previous experiments showed that this temperature allowed a faster growth of the different bacteria. Each point of the gradient griddle was inoculated with 2 µl of culture. The tests were repeated in triplicate for each bacterial strain in different gradient plates. Isolates were scored as resistant if they can grow along the gradient griddle. In order to confirm the results obtained by this procedure, bacteria were then grown on TSA plates containing different nominal concentration of TBT (0, 0.025, 0.05, 0.075, 1, 1.5, 2, 3 mM). Plates were incubated at 26 °C for 20 h in the dark.

Two isolates, one very resistant (*Aeromonas veronii* - Av27) and one sensitive (*Shewanella algae/putrefaciens* - Av35) were selected to compare their growth behaviour in liquid medium containing different TBT concentrations (0, 0.5, 1.5 and 3 mM). Growth occurred in TSB at 250 rpm, 26 °C for 72 h, and was recorded as a change of optical density at $A_{600\text{ nm}}$.

2.3. Memory response to progressive TBT exposure

Bacteria that have the ability to grow on 0.05 mM of TBT were successively transferred to TSA plates containing increasingly higher TBT concentrations (1, 1.5 and 2 mM). Transfer to a new media occurred after 48, 72 and 96 h, as long as growth was observed. Plates were incubated at 26 °C in the dark.

2.4. Antibiotic and heavy-metal sensitivities

Antibiotics to be tested were selected according to the Clinical Laboratory Standard Institute (CLSI) (Clinical Laboratory Standard Institute, 2005). The tests were performed by the Vitek[®] system (bioMérieux, Hazelwood, MO, USA). Those included: amikacin ($\leq 2 \mu\text{g ml}^{-1}$), aztreonam ($\leq 8 \mu\text{g ml}^{-1}$), cefepime ($\leq 4 \mu\text{g ml}^{-1}$), ceftazidime ($\leq 8 \mu\text{g ml}^{-1}$), ciprofloxacin ($\leq 0.5 \mu\text{g ml}^{-1}$), gentamicin ($\leq 0.5 \mu\text{g ml}^{-1}$), imipenem ($\leq 4 \mu\text{g ml}^{-1}$), netilmicin ($\leq 4 \mu\text{g ml}^{-1}$), pefloxacin ($\leq 1 \mu\text{g ml}^{-1}$), piperacillin ($\leq 8 \mu\text{g ml}^{-1}$), piperacillin/tazobactam ($\leq 8 \mu\text{g ml}^{-1}$), ticarcillin ($\leq 16 \mu\text{g ml}^{-1}$), ticarcillin/clavulanic acid ($\leq 16 \mu\text{g ml}^{-1}$), tobramycin ($\leq 0.5 \mu\text{g ml}^{-1}$) and trimethoprim/sulfamethoxazol ($\leq 10 \mu\text{g ml}^{-1}$). Results were interpreted according to the CLSI criteria (Clinical Laboratory Standard Institute, 2005).

Heavy-metal resistance was tested using gradient plates by the incorporation of the selected metal in TSA medium (as previously described in section 2.2). Metals and respective concentrations selected were based on previous determinations carried out in Ria de Aveiro (Monterroso et al., 2003). Heavy-metal stock solutions were prepared in distilled water with the following molarities: Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 M; Zn as ZnCl_2 , 0.1 M; Cd as CdCl_2 , 0.1 M; Hg as HgCl_2 , 0.1 M. Final concentrations of each of the heavy-metals were: $75 \mu\text{g ml}^{-1}$ of HgCl_2 , $3483 \mu\text{g ml}^{-1}$ of

CuSO₄.5H₂O, 200 µg ml⁻¹ of ZnCl₂ and 56000 µg ml⁻¹ of CdCl₂. Bacteria grew on TSB at 26 °C and 250 rpm (Sanyo incubator) until they reached an optical density (OD_{600 nm}) of 1 (approximately 10 h of incubation). Each point of the gradient griddle was inoculated with 2 µl of culture. Tests were performed in triplicate for each bacterial strain in independent gradient plates. Plates were incubated at 26 °C for 20 h in dark conditions. Resistance profile was defined in function of growth observed along the gradient griddle.

2.5. TBT degradation by *Aeromonas veronii* Av27

To evaluate the ability of *A. veronii* Av27 to degrade TBT, a preliminary experiment was designed as follows: 100 ml of TSB medium containing 50 µM of TBT were inoculated with 1 ml of an exponential phase culture of *A. veronii* (approximately 10⁸ cells ml⁻¹) and incubated for 24 h in an orbital shaker (Sanyo) at 26 °C, 250 rpm, in the dark (to avoid photo degradation of TBT) (corresponding to sample II). A control sample, consisted of non-inoculated medium, put in the same conditions (corresponding to sample I).

After the incubation period, the bacterial culture was centrifuged at 8000 x g, for 10 min; cells were lyophilised and kept at -70 °C until analysis (sample IIB); the resulting supernatant was also lyophilised and kept at -70 °C until analysis (sample IIA). Control sample was subjected to lyophilisation prior to storage at -70 °C, until analysis (sample I). Three independent replicates were performed for each experimental condition.

Organotin determinations were performed by gas chromatography/mass spectrometry (GC-MS). The GC-MS method used is described in detail elsewhere (Szpunar et al., 1996; Quintela et al., 2000). In summary, three replicates of 0.1 g lyophilized cells/supernatant were digested (microwave assisted) with tetramethylammonium hydroxide. pH was adjusted to 5, sodium

tetraethylborate and isooctane, containing tetrabutyltin as an internal standard, were added successively. After microwave treatment, the organic phase was recovered and analyzed by GC-MS. Recoveries of spiked organotins were, on average, 90% TBT, 98% DBT, 51% MBT. The determinations were performed at Servicios Xerais de Apoio á Investigación (Universidade da Coruña).

2.6. TBT utilization as carbon source and cell viability determination

Growth of *A. veronii* Av27 on TBT as the primary carbon source was evaluated as follows: 1 ml of an exponential phase culture of *A. veronii* (approximately 10^8 cells ml⁻¹) was inoculated in Minimal Medium- MM - (KH₂PO₄ 1.0 g, MgSO₄·7H₂O 200.0 mg, FeSO₄·7H₂O 10.0 mg, CaCl₂ 10.0 mg, vestigial elements (Mn, Mo, Cu, Co, Zn) as organic salts, 0.02 mg of each, NH₄Cl 1.0 g, distilled water to 1l, pH 6.0) containing different concentrations of the TBT (0, 1.5, 3 mM) and incubated at 26 °C and 250 rpm (Sanyo incubator shaker) for 72 h, in the dark.

TBT stock solution is prepared in ethanol; therefore, a control was prepared using the same volume of ethanol (30 ml l⁻¹), to assure that the cell growth was supported by TBT. At 3 h intervals, 1 ml aliquots were withdrawn from the culture. Optical density was monitored at 600 nm (A_{600 nm}), using a Lambda 16 Spectrophotometer (Cary 50B.O UV-visible spectrophotometer-Varian). Three independent replicates were performed for each experimental condition.

Bacterial cells viability was determined, using 1 ml of bacterial cultures (approximately 10^8 cells ml⁻¹) grown in MM with (3 mM TBT) and without TBT. Three microliters of the dye mixture (propidium iodide and SYTO 9, LIVE/DEAD® BacLight™ of bacterial viability kit, Molecular Probes) was mixed with water to a final concentration of 30 µM propidium iodide and 5.01 µM Syto 9. The samples were incubated for 15 min in the dark at room temperature (RT), according

186 to the manufacturer's instructions, filtered through 0.2 μ m black polycarbonate membranes
187 (Poretics) and mounted between a slide and cover slip with BacLight mounting oil (Molecular
188 Probes). The green (intact membrane) and red (damaged membrane) were enumerated after 18-24
189 h using epifluorescence microscopy. Ten microscope fields or at least 200 cells were enumerated
190 for each of 3 replicate samples.

3. RESULTS

Bacterial isolation, identification and TBT sensitivity

Fifty morphologically different strains were isolated from water and sediment samples collected at Ria de Aveiro. Forty six of those isolates were identified, whenever possible, to the species level (table 1). Four strains could not be identified. Growth along a TBT gradient, in solid medium showed that the sensitivity of the different strains to this compound was very heterogeneous (table 1). No consistency was observed at the species level, among the isolates in respect to their TBT resistance profile. At the genera level similar results were observed; for instance, in *Bacillus* (Av1, 2, 3 and 4) and *Brevibacterium* (Av5 and 6), where species belonging to same genera have a different behaviour in respect to TBT resistance. Among the isolates, 16 strains could grow on concentrations of up to 3 mM. Those strains belong to various genera. Some strains could not grow at all in the gradient plates. Nevertheless this may be due to some diffusion of the compound through the agar which means that TBT concentration never equals zero in the gradient plate and, therefore, growth is impaired even when the concentration is apparently close to zero. Thirteen strains were able to grow at intermediate TBT concentrations (0.025 mM - 0.05 mM TBT) and, once again, significant divergent resistance profiles were observed in bacteria belonging to the same genera. Overall, the percentage of Gram positive bacteria isolated that were sensitive to TBT (no growth) was 43.5 % whilst Gram negative bacteria represented only 7.4 %. Although the samples were collected at the same site in Ria de Aveiro, no consistency was observed between bacteria TBT resistance profile and nature of the sample (water or sediment). Also, different resistance patterns were observed among bacteria isolated from water and from sediment.

Despite the fact that several bacterial isolates showed resistance to high TBT concentrations (3 mM), only one particular isolate, Av27, identified as *Aeromonas veronii* biovar *sobria*, was selected for further studies, because it possesses 4 plasmids, that might codify for TBT resistance, and also because its total DNA was more efficiently digested than the DNA of other resistant isolates, which would be a crucial step for the molecular studies.

Growth behaviour of Av27 on liquid medium with TBT

Figure 1 compares growth of *Aeromonas veronii* biovar *sobria* (Av27), in liquid culture media containing different TBT concentrations, with that of *Shewanella algae/putrefaciens* (Av35-sensitive to TBT). It can be observed that strain Av27 can grow in the presence of high concentrations of TBT (up to 3 mM), while Av35 could not grow at the lowest concentration tested (0.5 mM).

‘Memory response’ to progressive TBT exposure

After the first screening for the isolation of TBT resistant strains, it was observed that only two Gram positive and seven Gram negative isolates could grow in 2 mM of TBT (table 1). However we noticed that, if those isolates that could not grow up to this concentration were successively transferred to increasingly higher TBT concentrations (up to 2 mM), the number of isolates increased to five Gram positive isolates and 14 Gram negative isolates. These results showed that, *in vitro*, bacteria can adapt to higher TBT concentrations, suggesting the existence of a ‘memory response’ to previous exposure.

Antibiotic and heavy-metal sensitivity of isolate Av27

A. veronii Av27 showed to be sensitive (no growth observed) to all the antibiotics tested (table 2). Considering heavy-metal resistance pattern, *A. veronii* Av27 can grow up to 10 $\mu\text{g ml}^{-1}$ of HgCl_2 , 498 $\mu\text{g ml}^{-1}$ of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 86 $\mu\text{g ml}^{-1}$ of ZnCl_2 and up to 56000 $\mu\text{g ml}^{-1}$ of CdCl_2 (table 2).

TBT degradation by *Aeromonas veronii* Av27

Figure 2 represents organotin degradation determined when *A. veronii* Av27 was grown in TSB over a period of 24 h. Likewise table 3 shows the GC-MS values of organotin analysis. Sample I corresponds to non-inoculated culture media that was kept in the same conditions of incubation as the inoculated sample (sample II). It can be seen that no TBT degradation occurred under these conditions and mass spectrometry values for DBT and MBT were below the detection limit (table 3). Therefore, it can be assumed that 100 % of TBT remained in these samples. Samples IIA and IIB correspond to the culture supernatant of a 24 h growth of *A. veronii* and to the pelleted cells, respectively. In sample IIA, DBT was detected (120 $\mu\text{g ml}^{-1}$) and TBT and MBT values were below the detection limit, suggesting TBT degradation has occurred and that the resulting compounds of degradation are extruded from the cell and released to the culture media. Sample IIB reveals the amount of organotins that are present in the cells. The values correspond to about 89 % of TBT (0.32 $\mu\text{g ml}^{-1}$), 10 % DBT (0.037 $\mu\text{g ml}^{-1}$), and only 1 % to MBT (0.0042 $\mu\text{g ml}^{-1}$). These results suggest that either TBT remains adhered to the cells or part of it, that is degraded, remains in the cells.

TBT a carbon source for *Aeromonas veronii* Av27

Figure 3 shows the growth of *A. veronii* in appropriate culture medium in the presence of increasingly higher concentrations of TBT (0, 1.5, 3 mM). It can be observed that at a higher

TBT concentration (3 mM), the culture reached an optical density $A_{600\text{ nm}}$ of 0.7 after 72 h. When TBT concentration is lower (1.5 mM), lower optical density values are observed ($A_{600\text{ nm}}$ of 0.5) at the same time interval. Similarly, when a control growth curve is done in the presence of ethanol, the TBT solvent, bacteria growth was slower ($A_{600\text{ nm}} = 0.3$), suggesting that in fact, *A. veronii* uses TBT as carbon source for metabolic activities.

Also, when *A. veronii* is grown in MM the total cell counts, as revealed by microscopy, were lower ($120 \times 10^6 \text{ ml}^{-1}$) when compared to the total cell number observed when bacteria is growing in the presence of TBT ($160 \times 10^6 \text{ ml}^{-1}$). Although the differences observed were not significant it is possible to suggest that TBT stimulates an increment in the cell number.

Microscope observations showed that when bacteria grow in MM without TBT, cells are bigger and appear dispersed in the culture medium, nonetheless when bacteria grow in presence of TBT, the cells look smaller and appear to aggregate in the culture medium.

Total viable counts showed that when *A. veronii* was grown in MM (without TBT), the number of viable cells is lower (22 %) than when bacteria was grown in MM with 3 mM TBT (45 %).

4. DISCUSSION

In the present study the gradient plate assay proved to be very useful method to detect different TBT resistance patterns among the bacterial isolates collected in Ria de Aveiro, allowing for the selection of a wide variety of cultivable bacterial genera exhibiting a TBT resistant phenotype.

Given the important role played by bacteria in biogeochemical processes, these bacteria consortia play an important role in estuarine systems.

A significant difference was observed between isolates belonging to the same genera. For instance, in the genera *Bacillus*, two strains *Bacillus cereus* (Av2) and *Bacillus firmus* (Av3) showed different tolerance to TBT (Table 1), despite the fact that they were both isolated from sediment samples. Similar observations were reported by Jude and co-workers (1996) in *Pseudomonas* species, where bacteria isolated from Arcachon harbor showed different TBT tolerance ranging from 0.07 and 3 mM.

In the present study, no significant correlation was observed between strains isolated from the more polluted area (water) and strains collected at the less polluted area (sediment). These findings were also observed by other researchers, suggesting that other factors rather than the presence of TBT select for a resistant population (Wuertz et al., 1991; Jude et al., 1996).

Ramamoorthy and Kushner (1975) suggested that medium-organometal interactions may affect metal toxicities. Nevertheless, despite the fact that TBT could act as a chelating agent sequestering carbon /nitrogen sources when a rich medium is used (e.g. TSB), this could not be observed in the present study, as bacteria retained the same TBT profile independently of the culture media used (MB, MBA, TSB, TSA).

Furthermore, we found that despite the heterogeneity observed in the TBT resistance profile of Gram negative and Gram positive bacteria, we found that the former are generally more resistant

to TBT. These findings were also observed by other authors and could be related with the different architecture of bacterial cell walls which, in Gram negatives, could promote the ability to immobilize metal ions (Beveridge and Koval, 1981). Nonetheless, five Gram positive strains were shown to be highly resistant to TBT. These findings suggest that, not only the external membrane but also cytoplasmic membrane and/or intracellular level mechanisms might be involved in the resistance, as suggested by White et al. (1999).

Successive transfer of the isolated bacteria to progressively higher TBT concentrations resulted in an increased resistance to TBT. These results are in accordance with those reported by Dahllöf and co-workers (2001), where in a marine sediment community, progressive exposure to higher TBT concentrations showed an increase in bacterial resistance. Also, Blanck and Dahl (1996) reported that exposure to a toxicant for some time can increase tolerance to that particular toxicant. Therefore, according to our observations we proposed that this fact could reflect an inducible ‘memory response’ mechanism to TBT/toxicant exposure. Nonetheless, the relation between the ‘memory response’ to TBT concentration and the resistant bacteria on a given natural environment could not be made because other factors, for instance the presence of other toxic compounds, could influence the ‘memory’ mechanism response.

Due to the relevant aspects previously highlighted, *A. veronii* Av27 was selected for a series of studies which were conducted in the present work. This is the first report of an *Aeromonas* bacterium exhibiting high resistance to TBT. Until now, only members from *Pseudomonas* and *Alteromonas* genera were associated with TBT resistance. In those cases both strains were used to investigate the TBT resistance mechanism (Suzuki and Fukagawa, 1995; Jude et al., 2004; Roy, Dubey and Bhosle, 2004), suggesting the existence of different mechanisms in those two genera.

A. veronii Av27 is sensitive to various antibiotics and did not show high tolerance to mercury. However, according to the resistant pattern observed in several environmental strains (e.g. *Pseudomonas*, *Proteus*, *Xanthomonas*, *Alteromonas*, *Aeromonas*, and Enterobacteriaceae) studied by De and co-workers (2003), *A. veronii*, seems to be resistant to cadmium, copper and zinc, despite the fact that the values detected in Ria de Aveiro (Monterroso et al., 2003) are higher than those used by these authors. These results are quite intriguing, as we expected that *A. veronii* Av27 would also be resistant to several classes of antibiotics, considering that Ria de Aveiro is a reservoir of several resistance determinants (e.g. antibiotics and antibiotic gene cassettes) that are released from industrial and domestic effluents (Henriques et al., 2006). Nevertheless, Jude et al. (2004) identified in *Pseudomonas stutzeri*, a multidrug efflux pump, belonging to the resistance-nodulation-division (RND) family that conferred resistance to some toxic compounds and different classes of antibiotics.

Degradation of TBT in DBT occurred under laboratory conditions by *A. veronii* Av27, in 24 h. Also, it seems that TBT is taken up by the cells, possibly, into siderophores structures, and it is gradually exported, as DBT, after degradation inside the cell. Despite the fact that debutylation of TBT results in less lipophilic compounds, due to cell activity, it appears that these compounds are still bound to the cell membrane lipids. Previously, Suzuki and Fukagawa (1995) reported that in a resistant strain, after 1 h of growth, the uptake of TBTCI was registered. However, during the incubation, a decrease of TBTCI uptake occurred probably due to efflux of the compound. These observations corroborate our results. Also, Roy et al. (2004) reported that a *Pseudomonas* sp. was able to use TBT as a source of carbon only at concentrations of up to 2 mM. In the present study, TBT was used as a source of carbon up to a concentration of 3 mM by *A. veronii* Av27, a concentration never reported before. Bacterial viability analysis showed that despite the higher

number of cells observed when TBT is present in the culture medium, meaning that bacteria are using the available TBT, cells were smaller and appeared aggregated. This could be explained as an energy saving mechanism exhibited by the bacteria when exposed to TBT for long periods. Despite their resistance to TBT bacteria cells are growing under stress conditions, due to the scarcity of nutrients. This was also observed and suggested by other authors for cells growing under stress conditions in the presence of other toxic compounds (Dykhuizen et al., 1987). The results so far obtained suggest that *A. veronii* Av27 developed a physiological mechanism that gives the bacteria the ability to use TBT as a carbon source and to degrade it to less toxic compounds, conferring the advantage of resistance to high TBT concentrations.

5. CONCLUSION

Given its ability to degrade and utilize TBT as carbon source, *A. veronii* Av27 constitutes an important organism that could be used as a tool for genetic studies. These studies are already in progress with the aim of elucidating the mechanism behind TBT resistance at the genetic level. Furthermore, this strain might have the potential to be developed as a biosensor to detect TBT in the environment. Ultimately, *A. veronii* could be applied in the natural remediation of TBT contaminated areas.

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FIGURES CAPTIONS

Figure 1. Growth of *A. veronii* Av27 in TSB medium with 0, 0.5, 1.5, 3 mM TBT and strain Av35 (sensitive to TBT) in TSB medium with of 0.5 mM TBT. Growth occurred at 26 °C for 72 h and 250 rpm, and it was monitored as a change of absorbance at 600 nm. Standard deviations are indicated in the graph.

Figure 2. Percentage of organotins detected by mass spectrometry. Sample I: TSB culture medium with 50 µM of TBT, incubated for 24 h, at 26 °C, 250 rpm, in the dark; Sample II: *A. veronii* Av27 culture on TSB medium with 50 µM TBT, incubated for 24 h, at 26 °C, 250 rpm; supernatant corresponds to Sample IIA and the pellet corresponds to Sample IIB.

Figure 3. Growth of *A. veronii* Av27 in Minimal Medium (MM) with different concentrations of TBT (0, 1.5 and 3 mM) at 26 °C for 72 h and 250 rpm, in the dark. “MM + ethanol” corresponds to the negative control. Growth was monitored as a change of absorbance at 600 nm. Standard deviations are indicated in the graph.

Table 1 – Growth of bacterial strains isolated from sediment and water in gradient agar plate assay to evaluate the effect of TBT.

Gram positive bacteria				Gram negative bacteria			
Strain code	Identification	TBT resistance pattern	Source	Strain code	Identification	TBT resistance pattern	Source
Av1	<i>Bacillus amyloliquefaciens</i>	-	Sediment	Av24	<i>Acinetobacter lwoffii/junii</i>	++	Sediment
Av2	<i>Bacillus cereus/thuringiensis</i>	++	Sediment	Av25	<i>Aeromonas hydrophyla</i>	++	Sediment
Av3	<i>Bacillus firmus</i>	-	Sediment	Av26	<i>Aeromonas salmonicida</i>	++	Sediment
Av4	<i>Bacillus megaterium</i>	-	Sediment	Av27	<i>Aeromonas veronii biovar sobria</i>	++	Sediment
Av5	<i>Brevibacterium spp. (1)</i>	++	Sediment	Av28	<i>Pseudoalteromonas sp.</i>	++	Sediment
Av6	<i>Brevibacterium spp. (2)</i>	+-	Sediment	Av29	<i>Citrobacter sp.</i>	++	Sediment
Av7	<i>Cellulomonas sp.</i>	-	Sediment	Av30	<i>Enterobacter intermedius</i>	++	Sediment
Av8	<i>Corynebacterium auris</i>	-	Sediment	Av31	<i>Enterobacter intermedius</i>	++	Sediment
Av9	<i>Corynebacterium auris</i>	-	Sediment	Av32	<i>Stenotrophomonas maltophilia</i>	+	Sediment
Av10	<i>Corynebacterium propinquum</i>	+-	Sediment	Av33	<i>Acinetobacter</i>	+	Sediment
Av11	<i>Kurthia gibsonii</i>	+-	Sediment	Av34	<i>Vibrio tasmaniensis</i>	+-	Water
Av12	<i>Kurthia zopfii</i>	++	Sediment	Av35	<i>Shewanella algae/putrefaciens</i>	-	Water
Av13	<i>Listeria grayi</i>	++	Sediment	Av36	<i>Pseudoalteromonas sp.</i>	+	Water
Av14	<i>Microbacterium spp.</i>	+-	Sediment	Av37	<i>Shewanella sp.</i>	-	Water
Av15	<i>Oersinia xanthineolytica</i>	-	Sediment	Av38	<i>Pseudoalteromonas sp.</i>	+	Water
Av16	<i>Propioni avidum</i>	-	Sediment	Av39	<i>Pseudoalteromonas sp.</i>	++	Water
Av17	<i>Turicella otitidis</i>	++	Sediment	Av40	<i>Pseudoalteromonas halophyla</i>	+	Water
Av18	<i>Micrococcus luteus</i>	-	Sediment	Av41	<i>Pseudoalteromonas sp.</i>	+-	Water
Av19	<i>Staphylococcus xylosus</i>	+-	Sediment	Av42	<i>Arctic sea ice bacterium</i>	+	Water
Av20	<i>Aureomonas spp.</i>	-	Water	Av43	<i>Pseudomonas fluorescens/putida</i>	++	Water
Av21	<i>Arcano pyogenes</i>	+-	Water	Av44	<i>Shewanella sp.</i>	+-	Water
Av22	*	+	Water	Av45	<i>Vibrio splendidus</i>	+	Water
Av23	*	+	Water	Av46	<i>Shewanella violacea</i>	+	Water
				Av47	*	++	Water
				Av48	<i>Vibrio sp. Da2</i>	+-	Water
				Av49	*	+-	Water
				Av50	<i>Vibrio sp. OC26</i>	+	Water

(*) bacteria not identified
 (-) no growth
 (+-) growth between 0.025 mM and ~ 0.05 mM TBT
 (+) growth up to ~ 2 mM TBT
 (++) growth up to ~ 3 mM TBT

461

462 Table 2 – Antibiotic and heavy-metal susceptibilities of *A. veronii* Av27.

Response to ^a (MIC values in $\mu\text{g ml}^{-1}$)															Maximum Resistance (up to $\mu\text{g ml}^{-1}$)			
AMK ≤ 2	ATM ≤ 8	FEP ≤ 4	CAZ ≤ 8	CIP ≤ 0.5	GEN ≤ 0.5	IMI ≤ 4	NTL ≤ 4	PEF ≤ 1	PIP ≤ 8	TZP ≤ 8	TIC ≤ 16	TCC ≤ 16	TOB ≤ 0.5	SXT ≤ 10	HgCl ₂	CuSO ₄ ·5H ₂ O	ZnCl ₂	CdCl ₂
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	10	498	86	56000

463

464 ^aAMK, Amikacin; ATM, Aztreonam; FEP, Cefepime; CAZ, Ceftazidime; CIP, Ciprofloxacin; GEN, Gentamicin; IMI, Imipenem; NTL,

465 Netilmicin; PEF, Pefloxacin; PIP, Piperacillin; TZP, Piperacillin/tazobactam; TIC, Ticarcillin; TCC, Ticarcillin/clavulanic acid; TOB,

466 Tobramycin; SXT, Trimethoprim/sulfamethoxazol.

467 S: Sensitive (i.e. no growth above threshold concentration)

468 Maximum Resistance: (maximum concentration at which growth is still observed)

Table 3. Organotin determinations by GC-MS. Sample I: TSB culture medium with 50 μM of TBT (24 h incubation); Sample IIA: supernatant of TSB medium with 50 μM TBT inoculated with *A. veronii* Av27 (24 h culture); Sample IIB: pellet of TSB medium with 50 μM TBT inoculated with *A. veronii* Av27 (24 h culture). bdl: below detection limit. Detection limits: monobutyltin (MBT) = 0.03 ng ml^{-1} ; dibutyltin (DBT) = 0.2 ng ml^{-1} ; tributyltin (TBT) = 0.07 ng ml^{-1} .

		Organotin determinations by GC-MS		
		MBT	DBT	TBT
Samples	I	bdl	bdl	130 $\mu\text{g ml}^{-1}$
	IIA	bdl	120 $\mu\text{g ml}^{-1}$	bdl
	IIB	0.0042 $\mu\text{g ml}^{-1}$	0.037 $\mu\text{g ml}^{-1}$	0.32 $\mu\text{g ml}^{-1}$

Figure 1

Figure 2

Figure 3