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*sug*E: A gene involved in tributyltin (TBT) resistance of *Aeromonas molluscorum* Av27

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The mechanism of bacterial resistance to tributyltin (TBT) is still unclear. The results herein presented contribute to clarify that mechanism in the TBT-resistant bacterium *Aeromonas molluscorum* Av27. We have identified and cloned a new gene that is involved in TBT resistance in this strain. The gene is highly homologous (84%) to the *Aeromonas hydrophila-sug*E gene belonging to the small multidrug resistance gene family (SMR), which includes genes involved in the transport of lipophilic drugs. In Av27, expression of the Av27-*sug*E was observed at the early logarithmic growth phase in the presence of a high TBT concentration (500 μM), thus suggesting the contribution of this gene for TBT resistance. *E. coli* cells transformed with Av27-*sug*E become resistant to ethidium bromide (EtBr), chloramphenicol (CP) and tetracycline (TE), besides TBT. According to the Moriguchi logP (miLogP) values, EtBr, CP and TE have similar properties and are substrates for the *sug*E-efflux system. Despite the different miLogP of TBT, *E. coli* cells transformed with Av27-*sug*E become resistant to this compound. So it seems that TBT is also a substrate for the SugE protein. The modelling studies performed also support this hypothesis. The data herein presented clearly indicate that *sug*E is involved in TBT resistance of this bacterium.

Key Words——*Aeromonas molluscorum* Av27; resistance; small multidrug resistance proteins; *sug*E; tributyltin

Introduction

Tributyltin (TBT) is one of the most toxic xenobiotics deliberately introduced into the environment by Man (Goldberg, 1986). It has been employed in a variety of industrial processes and it is subsequently discharged into the environment (Gadd, 2000). Production, use and export of TBT have been prohibited in developed countries since the 1990s by the International Maritime Organization (IMO). The European Union (EU) introduced Directive 2002/62/EC, which forbids the use of TBT in any boat after 2008. However, considering the long half life of TBT, it is likely that it will remain in the water column and sediments for up to 20 years after the cessation of TBT inputs into the environment. TBT is toxic to eukaryotes and prokaryotes. Examples of the effects of TBT in eukaryotes are the imposex (Barroso et al., 2000), which is the superimposition of male characters onto gastropod females, and immune system inhibition and endocrine disruption in humans

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(Dubey et al., 2006). In bacteria, the interference with biological membranes (Cooney and Wuertz, 1989; Cruz et al., 2012) and the inhibition of amino acid uptake and growth (Jude et al., 1996; Singh and Bragg, 1979) has been reported.

Some microorganisms are able to resist/degrade TBT (Cruz et al., 2007; Mimura et al., 2008; Suzuki and Fukagawa, 1995), this being a phenomenon of relevance to the environmental cycling of this compound. Considering TBT tolerance/resistance of bacteria, various possible mechanisms have been proposed: (i) TBT efflux systems (Fukagawa and Suzuki; 1993; Hernould et al., 2008; Jude et al., 2004); ii) transformation into less toxic compounds (dibutyltin, DBT and monobutyltin, MBT) by biotic and abiotic factors (Cruz et al., 2007; Pain and Cooney, 1998); (iii) degradation/metabolic utilization as a carbon source (Cruz et al., 2007; Kawai et al., 1998) and (iv) bioaccumulation into the cell without breakdown of the compound (Dubey and Roy, 2003). All together, these findings suggest that the mechanism of TBT resistance is quite complex and not unique among different bacterial strains. Nevertheless, so far, the resistance mechanism has not been fully elucidated in bacteria.

Aeromonas molluscorum Av27 was isolated from a moderately TBT-polluted marine sediment and it is highly resistant to TBT (up to 3 mM) (Cruz et al., 2007). Previous studies reported the degradation of TBT to DBT and MBT, and also its use as a carbon source by the Av27 strain (Cruz et al., 2007). A chromosomal fragment from this strain was isolated and cloned in *E. coli* HB101, conferring resistance to TBT to those cells (Cruz et al., 2010). The complete open reading frame (ORF) of that fragment has high homology with *A. hydrophila-sug*E, encoding the SugE protein. SugE belongs to the small multidrug resistance family (SMR) and it is involved in the transport of lipophilic drugs (Sikora and Turner, 2005). Herein, we show that the expression of Av27-*sug*E is triggered in response to TBT. The selective effect of resistance to xenobiotics, including to TBT, was also investigated. A theoretical model of Av27-SugE protein was obtained that contributes to the understanding of TBT transport into the bacterial cell.

Materials and Methods

Bacterial strains and growth conditions. *Aeromonas molluscorum* Av27 was isolated from sediment collected at Ria de Aveiro, Portugal (Cruz et al., 2007). The strain was maintained on Tryptic Soy Agar (TSA) plates (Merck, Germany) at 4°C. Growth in liquid culture of Av27 and *E. coli* HB101 occurred in Tryptic Soy Broth, TSB (Merck) at 26°C and in LB-Bouillon Miller broth, LB (Merck) at 37°C, respectively, and at 250 rpm. Cloning and sub cloning experiments were made in *E. coli* HB101 (Promega, USA). When necessary, the following selective agents were added to the growth media: ampicillin at 50 μg ml^{-1} and TBTCl (97%) (Fluka, Switzerland) at 0.05 to 3 mM. Hereafter, TBTCl will be referred as TBT. In all the experimental conditions where TBT was added, procedures were performed in the dark to avoid compound degradation.

Av27-sugE: a gene involved in TBT resistance. A genomic library of Av27 was constructed as described by Cruz et al. (2010). In one clone (clone 69) (Cruz et al., 2010), highly resistant to TBT, four ORFs were identified. Each ORF was amplified, subcloned in pUC19 vector and transformed in *E. coli* HB101.

TBT resistance of each subclone was confirmed by growth in LB medium supplemented with ampicillin (50 μg ml^{-1}), with (100 μM) and without TBT, at 37°C and 250 rpm. Growth was recorded as a change of optical density at 600 nm. pUC19 vector transformed into *E. coli* HB101 was used as the negative control. One subclone (ORF P6: Av27-*sug*E) showed a similar growth pattern to that of the original clone 69 and was therefore selected for further studies and characterization.

Expression analysis of Av27-sugE. Expression of Av27-*sug*E in *A. molluscorum* Av27 was investigated by Real Time-qPCR. The strain was grown in the presence of three different concentrations of TBT (0, 100 and 500 μM) at 26°C and 120 rpm. To improve the accuracy of the results, seven independent replicates were prepared for each condition. At an optical density (A_{600nm}) of 0.2 (early growth phase), 0.5 (mid growth phase) and 1 (late growth phase) the cells were pelleted by centrifugation and immediately frozen at −80°C. Total RNA was extracted with the RNeasy Mini kit (Qiagen, Germany) and DNA was completely removed with the Turbo DNA-free kit (Ambion, USA) according to the supplier's instructions. RNA concentration and integrity were determined with the Nanodrop (Thermo Scientific, USA) and by agarose gel electrophoresis. One microgram of RNA was used for reverse transcription (RT) reaction using the ReverTra Ace

qPCR RT Kit (TOYOBO, Japan). RT-qPCR was performed in the Applied Biosystems StepOne equipment, using the Fast SYBR Green Master Mix kit (Applied Biosystems, USA) according to the manufacturer's instructions. A control sample (with and without RT enzyme) was also included in the analysis. Specific primers (5′- ATG CCC TGG ATA TTG CTG CTC- 3′ and 5′- GGG TGA AAC CTT GGG TGT ATT TG- 3′) to amplify Av27-*sug*E gene were designed based on the Av27-*sug*E sequence, using the Primer3Plus program available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. The 16S rRNA gene was included as the internal standard. The relative quantity of gene expression was calculated using the ΔΔCt method (Biosystems, 2004). Statistical analysis was performed with GraphPad Prism5 (GraphPad Software, Inc., USA).

Modelling of Av27-SugE protein. A theoretical model of Av27-SugE protein using MODELLER (Sali and Blundell, 1993), employing the *Escherichia coli* EmrE cryomicroscopy structure (Ubarretxena-Belandia et al., 2003) as the template (protein databank code: 2I68), was constructed. The complete Av27-SugE protein and EmrE protein sequence share 34% sequence identity. Only the transmembrane helices of EmrE were experimentally determined; hence, only the transmembrane helices of Av27-SugE protein were modelled. This corresponds to the Av27-SugE protein residue segments: 3–20, 33–51, 57–79, 87–104, 107–124, 141–155, 161–180, 190–207. These segments correspond to the four transmembrane α-helices of each monomer. 20 models were generated using an initial alignment between the Av27-SugE protein and EmrE protein sequence carried out with MODELLER. The model with the lowest objective function (Sali and Blundell, 1993) was chosen and its quality was evaluated based on the stereochemistry of the generated model given by Procheck (Laskowski et al., 1993).

Determination of minimum inhibitory concentration (MIC). The resistance profiles of *E. coli* HB101 transformed either with Av27-*sug*E (*E. coli* HB101/Av27-*sug*E) or pUC19 vector (*E. coli* HB101/pUC19) to several chemicals were determined and compared. A broad range of toxic compounds, including representatives of cationic dyes, and neutral and anionic antimicrobials were used. The following compounds were tested: (i) cationic dyes: ethidium bromide (Applichem, USA, stock solution at 6 mg ml^{-1} prepared in distilled water), proflavine (Sigma-Aldrich, Germany, stock solution at 1 mg ml^{-1} prepared in distilled water), crystal violet (Merck, stock solution at 1 mg ml^{-1} prepared in absolute ethanol), cetylpyridinium chloride (Sigma-Aldrich, stock solution at 1 mg ml^{-1} prepared in distilled water); (ii) neutral antimicrobial: chloramphenicol (Biochemical, England, stock solution at 25 mg ml^{-1} prepared in absolute ethanol); (iii) other antimicrobial: tetracycline (Sigma-Aldrich, stock solution at 12.5 mg ml^{-1} prepared in absolute ethanol) and (iv) organotin compounds: TBT (stock solution at 0.1 M prepared in absolute ethanol), dibutyltin (Fluka, stock solution at 0.1 M prepared in absolute ethanol).

Serial twofold dilutions of the drugs were analyzed using the microwell dilution assay. Serial dilutions of each drug to be tested were prepared in LB medium containing ampicillin (150 μg ml^{-1}). After placing 100 μl of these dilutions into the 96-well plates, 100 μl of the bacterial cultures (*E. coli* HB101/Av27-*sug*E and *E. coli* HB101/pUC19), previously grown in LB medium containing ampicillin and with turbidity adjusted to 0.5 McFarland standard, were also added to the wells. Controls were prepared as above but no drug was added. The final volume in each well was 200 μl. The plates were covered with sterile plate sealers and incubated at 37°C for 24 h. Microbial growth was recorded at an optical density at 595 nm, with a microwell plate reader (iMark™ microplate reader, Biorad, USA). All the tests were performed in triplicate.

Statistical analyses. Statistical analyses of the data obtained in the gene expression and MIC experiments, were performed with GraphPad Prism 5 (GraphPad software, Inc., USA). One-way univariate analysis of variance model (ANOVA) was used, followed by the Tukey test to discriminate significant differences among all the tested conditions. A value of $p < 0.05$ was considered significant.

Nucleotide sequence accession number. The nucleotide sequence of ORF P6 (encoding Av27-*sug*E) is deposited in the GenBank under the accession # FJ225136.

Results and Discussion

Av27-sugE gene is involved in TBT resistance

Subcloning of ORF P6 (Av27-*sug*E) conferred phenotypic resistance to TBT to *E. coli* HB101 cells. This clone contained the pUC19 vector with an inserted fragment of 315 bp, whose deduced amino acid se-

quence has high homology (84% homology) to the SugE protein of *A. hydrophila*. SugE belongs to the small multidrug resistant family (SMR). Proteins belonging to this family are small (~12 kDa) integral inner membrane proteins. In the case of Av27-SugE, the hydropathy profile (data not shown) revealed that the protein is highly hydrophobic, like other members of the SMR family. These are characterized by spanning the cytoplasmic membrane as four transmembrane (TM) α-helices (Paulsen et al., 1996b) with short hydrophilic loops which make them very hydrophobic (Yerushalmi et al., 1996).

sugE expression in Av27 strain

Av27-*sug*E expression was investigated, in the Av27 strain, in the presence and absence of TBT. As shown in Fig. 1, when cells grow without TBT, the Av27-*sug*E gene is only expressed at a residual level (relative expression level lower than 5), independently of the growth phase of the cells. The same results were observed when cells were grown in the presence of 100 μM of TBT. However, different relative expression levels were observed when TBT was added at 500 μM. The expression of Av27-*sug*E was significantly higher at the early growth phase (Fig. 1A) ($p<0.05$), whereas at the mid and late growth phase (Fig. 1B, C), no significant differences were observed. These results indicate that the expression of Av27-*sug*E occurred at a high concentration of TBT and specifically increased at the early growth phase. At later growth phases no expression of Av27-*sug*E was observed. Since Av27 is highly resistant to TBT (Cruz et al., 2007) it appears that concentrations of 100 μM of TBT are not high enough to trigger the relative expression of Av27-*sug*E, although this concentration can inhibit the growth of other bacteria, for instance *Shewanella algae*/*putrefaciens* Av35 or *Bacillus firmus* Av3 (Cruz et al., 2007). Still, a higher concentration of TBT, for instance 500 μM, which is considered to be lethal for most bacteria (Cruz et al., 2007), seems to induce the expression of this gene, allowing bacteria to cope with the presence of the compound. These results confirm the role of Av27-*sug*E in the TBT resistance exhibited by this strain.

Since the relative expression of Av27-*sug*E was only observed in the early growth phase it seems that this rapid response to TBT is similar to that observed with iron-repressible outer membrane proteins of *Actinobacillus pleuropneumoniae*. In that strain a similar rapid expression induction was observed on iron-repressible outer membrane proteins within 15 to 20 min after establishment of iron starvation (Nielsen and Boye, 2005). Similarly, in another study with *E. coli*, growth phase-dependent expression was reported for other genes, namely *mdt*EF, encoding MdtEF protein, a major drug exporter that confers drug tolerance (Kobayashi et al., 2006). The *E. coli-sug*E gene encodes a chaperonin-related system whose composition might vary with temperature and growth phase. In that case, two expression models were proposed for *sug*E regulation permitting both i) constitutive and ii) stress-induced expression dependent on a still-undefined sigma factor (Greener et al., 1993). In the present study, Av27-*sug*E was constitutively expressed, and its expression was induced by the stress caused by TBT. Thus, it seems that both models are occurring simulta-

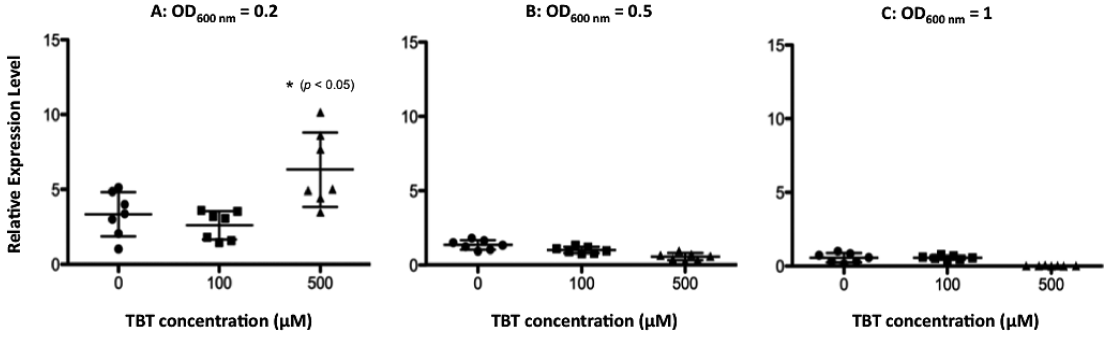

Fig. 1. Relative expression levels of Av27-*sug*E gene of *A. molluscorum* Av27.

Cells were grown without (0) and with TBT (100 and 500 μM). Samples were analyzed at different growth phases. A: early log phase (OD_{600nm}=0.2), B: mid log phase (OD_{600nm}=0.5) and C: late log phase (OD_{600nm}=1). Values were normalized against 16S rRNA, the internal standard and each sample was run in triplicate. * A value of $p<0.05$ was considered significant.

neously in Av27-*sug*E.

A model of Av27-SugE suggests a possible efflux of TBT

In order to perform TBT efflux and SMR substrate resistance experiments, we tried to generate a mutant containing disrupted Av27-*sug*E. Several attempts were made that involved transformation and transconjugation methods with no success. That was probably due to the intrinsic properties of the Av27 strain. Likewise, other authors reported the same impairment with *A. hydrophila* strains (Bello-López et al., 2012).

Nevertheless, we were determined to understand the role of SugE in TBT transport. With that purpose, the modelling of Av27-SugE protein (Fig. 2) was performed. The protein structure was based on the cryomicroscopy structure of EmrE from *E. coli* (Ubarretxena-Belandia et al., 2003) and from the latest reanalyzed EmrE X-ray data from Chen et al. (2007). Both experimental studies support an antiparallel homodimer quaternary structure forming a channel with TM helices TM1, TM2 and TM3; therefore, our theoretical model has also an antiparallel quaternary structural arrangement. Furthermore, only the TM helical structures were modelled due to the lack of structural data for the loop regions connecting the TM sections. The superposition of the Cα α-helix residues of our model with the EmrE cryomicroscopy structure gives an average root mean square displacement (RMSD) of 1.4 Å.

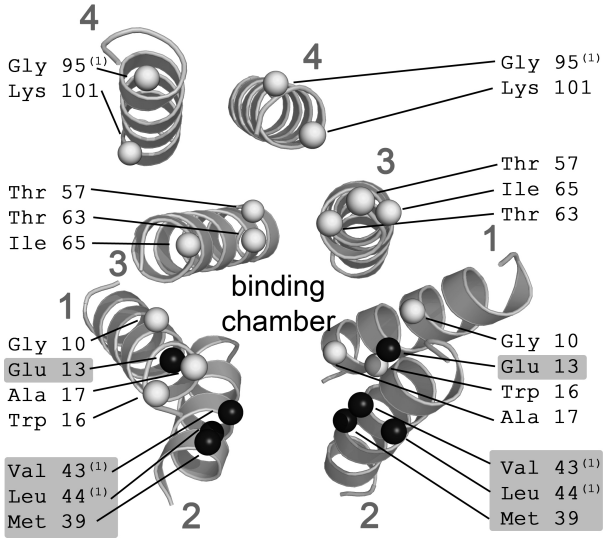

Fig. 2. Structural model of the Av27-SugE protein.

Location of the conserved and functional residues of Av27-SugE (in both monomers) that are specific to the SUG subclass (Bay et al., 2008). The Cα of the conserved residues of the SUG subclass is rendered as dark grey spheres. Location of the Glu 13 residue is also indicated on the figure and rendered as light grey spheres. Position of the essential residues for substrate binding studied by Son et al. (2003) (39, 43 and 44) is labelled on the figure and rendered as light grey spheres. Residues labelled with (1) indicate point mutations relative to SugE (*Citrobacter freundii*) protein described by Son et al. (2003). See text for details.

The precise location of the conserved and functional residues of the SUG protein subclass (represented by SugE from *Citrobacter freundii*) is shown on Fig. 2. Specific conserved SUG residues (position 39, 43 and 44) identified by Bay et al. (2008) are located facing the substrate binding chamber and interfacing the different TM helices. Av27-SugE protein does not have the same conserved residues, isoleucine 43 and alanine 44, that were found in SugE. In Av27-SugE protein, position 43 is occupied by a valine (equivalent nonpolar residue like isoleucine) and position 44 is filled with a leucine, a bulkier aromatic residue than alanine. Furthermore, Av27-SugE protein contains the highly conserved glutamate residue (Glu 13, see Fig. 2) located in the inner part of the binding chamber.

SMR family proteins are involved in the transport of lipophilic compounds, primarily quaternary ammonium compounds (QAC) as well as a variety of antibiotics (Chung and Saier, 2002; McIntosh et al., 2008), commonly used antiseptics, and detergents. These proteins are also responsible for drug efflux via an electrochemical proton gradient (Grinius and Goldberg, 1994; Yerushalmi et al., 1996) being classified as proton-dependent multidrug efflux systems (Paulsen et al., 1996a). The modelling studies of Av27-SugE herein performed allowed us to predict the predominance of nonpolar residues, along the α-helix structure of Av27-SugE protein, which is in agreement with its transmembrane location and the nonpolar characteristic of the binding chamber, essential for the protein association and the translocation of nonpolar solutes like TBT. Furthermore, it is also clear that some polar residues (including charged residues) are found in the transmembrane sections. The presence of charged residues is also essential for the proposed biochemical proton-dependent translocation mechanism of this small multidrug resistance protein family (Muth and Schuldiner, 2000). Av27-SugE contains also aromatic residues Y59 and W62 that are highly conserved in SMR proteins, which have been described as being involved in protein-drug interactions (Sikora

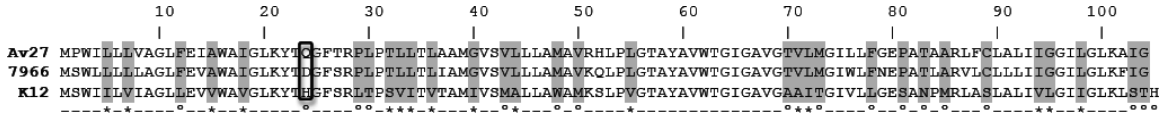

Fig. 3. Amino acid sequence alignment of the SugE of *A. molluscorum* Av27 (Av27), *A. hydrophila* 7966 (7966) and *E. coli* K12 (K12).

Grey boxes highlight the amino acids found in Av27 and 7966, but not in K12. Symbols: *, same chemical classification; °, different chemical classification; open box, amino acid at position 24, known to play a role in substrate specificity.

Table 1. Susceptibility of *E. coli* HB101/Av27-*sug*E and *E. coli* HB101/pUC19 to different classes of drugs.

Type of drug	Drug	MW	miLogP	MIC (μg ml^{-1})	
				E. coli HB101/Av27-*sug*E	*E. coli* HB101/pUC19
Cationic dyes	Ethidium bromide (EtBr)	394.34	0.304	422*	164
	Proflavine (PF)	209.25	1.269	31	23
	Crystal violet (CV)	408.03	3.196	14	6
	Cetylpiridinium chloride (CPC)	340.05	3.027	16	16
Neutral antimicrobial	Chloramphenicol (CP)	323.16	0.731	5*	2
Other antimicrobial	Tetracycline (TE)	444.44	−0.69	3*	1
Organotins	Tributyltin (TBT)	325.55	3.458	320*	53
	Dibutyltin (DBT)	303.88	2.601	7	5

* A value of $p < 0.05$ was considered significant. MW: molecular weight; miLogP: octanol-water partition coefficient logP.

and Turner, 2005).

Amino acid sequence alignment of Av27-SugE from *A. molluscorum* Av27, with other SMR proteins deposited in the databases, namely SugE from *A. hydrophila* 7966 (the closest homology, 84%) and SugE from *E. coli* K12 (ancestor of *E. coli* HB101, used in our cloning experiments) (Fig. 3) revealed some nucleotide and amino acids changes. An amino acid substitution was found at position 24, which has been reported to be related with substrate specificity in SugE and other SMRs (Son et al., 2003). This substitution may be involved in the substrate selection, namely on the binding of TBT. Some common amino acids were found in the SugE of Av27 and *A. hydrophila*, which were different from those found in *E. coli*. These might also be related to the intrinsic TBT resistance of some *Aeromonas* species and the susceptibility of the *E. coli* strain used in the present study.

Susceptibility to chemicals by Av27-sugE transformed E. coli

It has been shown that proteins belonging to the SMR family are involved in the export of drugs from bacterial cells, namely cationic drugs that are believed to translocate their substrates via a fairly hydrophobic transmembrane pathway (Mordoch et al., 1999). To help elucidate the involvement of the Av27-*sug*E gene in these mechanisms and also to evaluate the impact of this gene in the susceptibility of *E. coli* cells, MIC experiments were performed. The results shown in Table 1 revealed that the MICs of *E. coli* containing the Av27-*sug*E gene generally increased for all the drugs tested, when compared with *E. coli* HB101/pUC19. A significant increase ($p < 0.05$) was observed with EtBr (3-fold), CP (2.5-fold), TE (4-fold) and TBT (6-fold) (Table 1). These data indicate that the Av27-*sug*E gene affects the intrinsic multidrug resistance of *E. coli* HB101. EtBr is a hydrophobic cationic dye, which can be effluxed by SMR (Jack et al., 2000), and that was confirmed in the present study. SMR proteins like SugE appear to have a broad specificity spectrum that include not only the usual set of amphipatic cations, but also TE and CP (Jack et al., 2000). Chloramphenicol is a neutral antimicrobial that is fairly hydrophilic, suggesting its relatively poor membrane permeability (Lewis, 2001). Our results also showed that this compound is a substrate for Av27-SugE, possibly promoting its efflux across the cytoplasmic membrane. Con-

sidering TE, this drug forms a complex with divalent metals such as magnesium, which is excreted from the cell by efflux systems (Nonaka and Suzuki, 2002). The extrusion of TE by efflux is a common resistance mechanism. Accordingly, different tetracycline-pump families are known (Nonaka and Suzuki, 2002). In the present work, the resistance to TE increased due to the presence of the Av27-*sug*E gene, suggesting that *sug*E is possibly responsible for the efflux of the TE-Sn^{2+} complex.

Moriguchi LogP (miLogP) is a physicochemical factor of hydrophobicity and bioavailability of chemicals (Lipinski et al., 2001). Table 1 shows a comparison of the miLogP values with the increase of resistance. All of the tested compounds studied showed similar molecular size (ca. 200–400); nonetheless the miLogP values were separated into two groups. One group includes compounds with a significant increase of the MIC value (EtBr, CP, TE) and with lower miLogP values whereas the other group includes drugs with higher miLogP values. An exception was observed for TBT. Since TBT is a lipophilic and amphipathic compound with a higher miLogP value, it is expected that its action on bacteria will be similar to that of proflavine, crystal violet, cetylpiridinium chloride and dibutyltin. However, Av27-*sug*E might also recognize TBT as an efflux substrate, and therefore the exclusion of the compound from the cell will contribute to the TBT resistance observed in this strain.

Concluding Remarks

From the available literature a number of mechanisms contributing to TBT resistance have been described and seem to be present within the same strain and also among different strains and species (Dubey et al., 2006; Hernould et al., 2008; Kawai et al., 1998). Some of the mechanisms might be activated at different phases of the bacterial growth and/or when cells are exposed to different TBT concentrations. Likewise, in *A. hydrophila* it was suggested that in addition to the AheABC system, other RND-type efflux pumps contribute to the intrinsic drug resistance observed in this species (Hernould et al., 2008).

All together, our data support the involvement of the Av27-*sug*E gene in the resistance of *A. molluscorum* Av27 to TBT. The relative expression of the Av27-*sug*E gene is increased at the early growth phase when a high concentration of TBT is present. Additionally, Av27-*sug*E increased the intrinsic multidrug resistance of *E. coli* HB101 to EtBr, CP, TE and specifically to TBT. Moreover the aa substitution at position 24 of Av27-SugE supports the modelling data performed, reinforcing the specificity of Av27-SugE for TBT.

Acknowledgments

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