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SugE: protein involved in TBT resistance in *A. molluscorum* Av27

SugE: proteína envolvida na resistência ao TBT em *A. molluscorum* Av27

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Sónia Alexandra Leite Velho Mendo Barroso.

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palavras-chave TBT, Resistência, degradação, biorremediação, *A. molluscorum* Av27, Av27-SugE.

resumo

O uso extensivo de compostos organostânicos e a sua consequente descarga no ambiente levou à contaminação dos ecossistemas marinhos e de água doce. Vários estudos mostram que estes compostos estão envolvidos no fenómeno conhecido como "Imposex" em gastrópodes e que podem afectar outros organismos, incluindo os seres humanos. É assim, de grande importância, a remoção destes contaminantes dos ecossistemas.

Foi isolada e identificada uma espécie do género *Aeromonas* com elevada resistência ao tributilestanho (TBT). A estirpe, *Aeromonas molluscorum* Av27, é capaz de degradar o TBT em compostos menos tóxicos como o dibutilestanho (DBT) e o monobutilestanho (MBT). Foi demonstrado que nesta estirpe o gene *sug*E *A. molluscorum* Av27 (*sug*E-Av27) está implicado na resistência ao TBT. Este gene codifica para uma proteína homóloga à SugE, um membro da família das "small multidrug resistance" (SMR). Dada a possível aplicação de Av27 em processos de biorremediação, torna-se necessário aprofundar o estudo de todo o mecanismo de resistência nesta estirpe, bem como, optimizar o processo de avaliação da degradação do TBT pela estirpe Av27. O presente trabalho teve como objectivos: (i) verificar se, na presença de TBT, o aumento de expressão do gene *sug*E-Av27 se traduz num aumento da quantidade de proteína; (ii) clonar o gene *sug*E-Av27 num sistema de expressão em *Escherichia coli*, para posterior purificação da proteína; (iii) desenvolver e optimizar um método simples e rápido para detectar a degradação do TBT.

Não foi possível identificar a proteína SugE *A. molluscorum* Av27 (SugE-Av27) nos extractos proteicos. Contudo, da análise por espectrometria de massa, pode-se inferir que outros genes relacionados com a síntese proteica, glicólise e síntese de ATP estão a ser sobrexpressos em resposta à exposição ao TBT. Uma enzima, D-alanina-D-alanina ligase, envolvida na síntese da parede celular, parece estar também a ser sobrexpressa, indicando que um dos mecanismos de resistência possivelmente envolverá a manutenção da estabilidade da parede celular. Com vista à purificação da proteína em estudo, o gene *sug*E-Av27 foi inserido no vector pET24 e clonado em *E. coli* BL21 (DE3). Após quatro horas de indução com IPTG verificou-se um aumento da expressão da proteína SugE-Av27, por análise em Western blot.

Na tentativa de desenvolver um método rápido para detecção de TBT em laboratório e sendo *Micrococcus luteus* sensível ao TBT, utilizou-se está espécie indicadora em bioensaios de degradação do TBT pela Av27. Assim, verificou-se que após um período de 54h a toxicidade do TBT diminuiu, sendo então provável que este composto esteja a ser degradado pela estirpe Av27. No presente trabalho iniciaram-se alguns estudos que poderão, no futuro, contribuir para o esclarecimento do mecanismo de resistência /degradação ao TBT pela estirpe Av27. Contudo, mais estudos são necessários, nomeadamente no que refere à análise da expressão dos genes que estão a ser sub- e sobre-expressos em Av27 em resposta à exposição ao TBT e ainda no que refere ao bioensaio para avaliar a degradação do TBT por Av27.

keywords TBT, Resistence, degradation, bioremediation, *A. molluscorum* Av27, Av27-SugE.

abstract

The extensive use of organotin compounds and their subsequent discharge into the environment has led to contamination of marine and freshwater environments. Several studies shown that these compounds are involved in the phenomenon known as "imposex" in gastropods and may affect other organisms, including humans. It is thus of great importance, the removal of these contaminants in ecosystems. It was isolated species of the genus *Aeromonas* with high resistance to tributyltin (TBT). *Aeromonas molluscorum* Av27, is able to degrade TBT into less toxic compounds such as dibutyltin (DBT) and monobutyltin (MBT). It was demonstrated that the gene s*ug*E *A. molluscorum* Av27 (*sugE*-Av27), was linked to TBT resistance in this strain. This gene codes a protein homologous to SugE, a member of the small multidrug resistance protein family (SMR). Given the possible application of Av27 in bioremediation, it is necessary to study the whole mechanism of resistance and optimize the degradation of TBT in this Av27 strain. Thus, the objectives of this study were: (i) verify the over-expression of SugE *A. molluscorum* Av27 (SugE-Av27) protein in Av27 in the presence of TBT; (ii) clone Av27-*sug*E gene into an *Escherichia coli* expression system, which will allow further purification of the protein for future characterization studies; (iii) development and optimization of a simple and rapid method to evaluate the TBT degradation/toxicity.

In the protein extracts obtained it was not possible to identify the protein SugE-Av27. However, analysis by mass spectrometry, suggested that genes related to protein synthesis, glycolysis and ATP synthesis are being overexpressed in response to TBT exposure. An enzyme, D-alanine-D-alanine ligase, involved in cell wall synthesis, also appears to be overexpressed. Indicating that one of the resistance mechanisms can be related to the maintenance of the cell wall stability.

For the purification of the protein under study, the gene *sug*E-Av27 was inserted into the vector pET24 and cloned in *E.coli* BL21 (DE3). After four hours of induction with IPTG, there was an increased expression of SugE-Av27 protein indicated by Western blot analysis.

Micrococcus luteus was shown to be sensitive to TBT, thus can be applied as an indicator species in degradation bioassays of TBT by Av27. It was evidenced a decrease of the TBT toxicity to *M. luteus* after a period of 54h, therefore this compound is being degraded by Av27.

New perspectives opened up with this work in relation to TBT resistance mechanisms in Av27. To corroborate these results, further studies are needed including an analysis of the genes up- and downregulated in Av27 to TBT exposure and attempt some variations in the physicochemical parameters in the degradation bioassays.

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List of abbreviations

µg → microgram
µl → microliter
µM → micromolar
µm → micrometer
2,4-D → 2,4-dichlorophenoxyacetic acid
°C → Celsius degree

aa → amino acid
ACN → acetonitrile
Ala → alanine
ATP → adenosine-5'-triphosphate

BTEX → benzene, toluene, ethylbenzene and xylene

$CaCl_2$ → calcium chloride
Cl^+ → chloride ion

Da → dalton
DBT → dibutyltin
Ddl → D-alanine-D-alanine ligase
DDM → dodecyl maltoside
DTT → dithiothreitol

ECL → enzymatic chemiluminescence
EDTA → ethylenediaminetetraacetic acid

F → phenylalanine

g → gram

GC → gas chromatography
GEM → genetically engineered microorganisms
Glu-Fib → Glu-1-Fibrinopeptide B

h → hour
H^+ → hydrogen ion
HCl → hydrochloric acid
his → histidine

IMAC → immobilized-metal affinity chromatography
IMO → International Maritime Organization
IPTG → isopropyl β-D-1-thiogalactopyranoside

Kan → kanamycin
kDa → kiloDalton
kg → kilogram
k_{ow} → octanol-water partition coefficient
kV → kilovolt

l → liter

LA → Luria-Bertani broth agar
LB → Luria-Bertani Broth
LC → liquid chromatography

m → meter
M → molar
m/z → mass-per-charge
mA → milliamper

MALDI → matrix-assisted laser desorption/ionization
MB → marine broth
MBT → monobutyltin
mg → milligram
$MgCl_2$ → magnesium chloride
min → minute
ml → milliliter
mM → milimolar
mm → millimeter
mol → moles
MOPS → 3-(N-morpholino) propanesulfonic acid
MS → mass spectrometry

Na^+ → sodium ion
NaCl → sodium chloride
ng → nanogram
Ni^+ → niquel ion
nl → nanoliter
nm → nanometer

OD → optical density
OTC → organotin compounds

PAGE → polyacrylamide gel electrophoresis
PBC → polychlorinated biphenyls
PBS → phosphate buffered saline
PCR → polymerase chain reaction
PMSF → phenylmethanesulfonylfluoride
PSMR → paired small multidrug pumps
PVC → Poly(vinyl chloride)

QAC → quaternary ammonium compounds

rpm → rotations per minute

s → seconds
SDS → sodium dodecyl sulphate
SMP → small multidrug pumps
SMR → small multidrug resistance
Sn^{4+} → tin ion
SUG → suppressor of groEL mutation

TBST → tris-buffered saline and tween 20
TBT → tributyltin
TCE → trichloroethylene
TFA → trifluoroacetic acid
TM → transmembrane
TOF → time-of-flight
Tris → tris(hydroxymethyl)aminomethane
TSA → Tryptic soy agar
TSB → Tryptic soy broth

UV → ultraviolet

v/v → volume-per-volume

W → tryptophan
w/v → weight-per-volume

Y → tyrosine

A. Introduction

In a previous study, a bacterial strain, *Aeromonas molluscorum* Av27 (isolated from Ria de Aveiro, Portugal) was reported to be highly resistant to TBT (up to 3 mM). This strain showed to degrade TBT into the less toxic compounds, dibutyltin (DBT) and monobutyltin (MBT), and also to use it as a carbon source (Cruz *et al,* 2007).

Construction of a genomic library of Av27 strain, in *Escherichia coli*, revealed a gene involved in TBT resistance with high homology to the *sug*E gene (Cruz *et al*, 2010). The *sug*E gene encodes the SugE protein that belongs to the small multidrug resistance family, a lipophilic drugs transporter (Bay *et al*, 2008). Those results suggested that the SugE *A. molluscorum* Av27 (SugE-Av27) protein, encoded by *sug*E *A. molluscorum* Av27 (*sugE*-Av27) gene, is probably somehow involved in the transport of TBT in the Av27 strain (Cruz *et al*, 2010). Thus, it seemed important to study the role and characteristics of Av27-SugE protein.

The investigations carried by Cruz *et al.* (2007, 2010) suggest that Av27 can be potentially used in the bioremediation of TBT contaminated areas. Thus it is important to fully understand the mechanism of TBT resistance/degradation in this bacterium.

1. Organotin compounds: use and legislation

Some organotin compounds (OTC) like tributyltin (TBT) are widely used as fungicides, bactericides, pesticides, wood preservatives, PVC stabilizers, but are mainly used as additives in antifouling paints for boats (fig. 1). The extensive use and consequent discharge of these compounds into the environment, leads to marine and freshwater ecosystems contaminations. In fact, it has been shown that organotin compounds (OTC) are widespread in the environment and it is related to the worldwide decline of marine molluscs. The first report was the "Imposex"

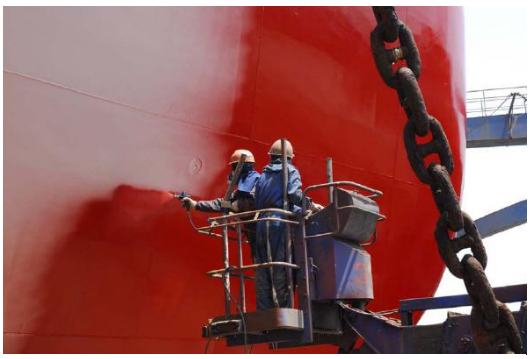


Fig. 1- Application of TBT containing antifouling paints in boats.

phenomenon in *Nucella lapillus,* characterized by the superimposition of male sex characteristics into female gastropods (Antizar-Ladislao, 2008; Blaber, 1970).

Since the 80´s when some reports demonstrated that these compounds affects non-targeted organisms, legislation that banned the use of TBT was applied to many countries (Konstantinou & Albanis, 2004). In 1982, France was the first country to ban the use of TBT-based antifouling paints from boats of less than 25m long (Alzieu et al, 1989). Till 1988, most European countries, North America, United Kingdom, Australia, New Zealand and Hong Kong implemented the same legislation (Antizar-Ladislao, 2008). The International Maritime Organization (IMO) adopted at 2001 a Convention that bans the use of TBT-based paints on ships, starting at 1 January 2003, and its total prohibition by 1 January 2008 (IMO, 2001). Unfortunately, only the prohibition of the use of these compounds will not solve the problem. TBT is already in the environment and will continue to be released in the environment, since there are lots of old boats that are painted with this antifouling paints, containing this toxic compound and, besides that, the legislation it is not universal. Development of new strategies to remove OTC from the environment is needed.

2. Tributyltin (TBT) properties

TBT is an organic compound characterized by the presence of covalent bonds between three carbon atoms and a tin atom (Sn^{4+}). It belongs to a family of compounds derivates from tin with the general formula R_nSnX_{4-n}, where X is an anion and R an alkyl group. The nature of X will influence the physicochemical properties of the different compounds. Tributyltin chloride is the specimen that is normally used in experiments for analysis of the OTC toxicity (fig. 2). Normally, the toxicity of the OTC is influenced more by the alkyl substitutes than the anionic

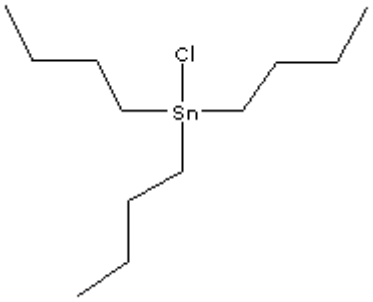


Fig. 2 - Chemical structure of tributyltin chloride.

substitute and shows less toxicity in this progression: $R_3SnX > R_2SnX_2 > R_1SnX_3 > RSnX_4$ (Dubey & Roy, 2003).

In the marine and freshwater ecosystems, TBT doesn´t remain for too long in the water column and adheres to bed sediments and organisms, due its high specific gravity near 1.2 kg l^{-1} at 20°C, a low solubility less than 10 mg l^{-1} at 20°C and pH 7.0, and a log K_{ow} values near 3.2. The affinity of OTC for adsorption to sediments is positively correlated to the extent of organo-substitution on the tin, such that increasing adsorption is seen for monobutyltin (MBT) < dibutyltin (DBT) < TBT (Landmeyer et al, 2004). Once released from the antifouling paint, TBT is rapidly adsorbed by the organic matter composed by bacteria, algae that enters in the food chain and eventually will contaminate higher organisms (Antizar-Ladislao, 2008).

3. Effects of TBT in microorganisms

Organotins are high lipophilic compounds that when in contact with the biota adheres immediately to the cells walls and membranes. TBT is a membrane-active compound and in biomembranes it is known that it can act as an ionophore, thus modifying energy transduction processes in bacteria, chloroplasts and mitochondria (Cooney & Wuertz, 1989; Wuertz et al, 1991).

The toxic effects of TBT in microorganisms are well documented and, as reviewed by Gadd (2000), it has been reported for all major groups (Table I). Cooney et al. (1989) studied the toxicity of organotin and organolead compounds in several yeasts and demonstrated that tributyltin had the highest toxicity level. These authors also showed that the toxicity of these compounds is influenced by the surrounding pH and salinity (Cooney et al, 1989). Supporting these findings, Laurence et al. (1989) also demonstrated that pH and salinity influenced the toxicity of organotins in the marine yeast *Debaryomyces hansenii*. These reports highlighted the importance of environmental factors in organotin toxicity (Gadd, 2000). For example, toxicity of the OTC is reduced in the seawater salinity levels, explained by the increase of Na^+ and Cl^- ions in the medium, which will possibly affect osmotic responses of the organisms,

including changes in intracellular compatible solutes and membrane composition, as reviewed by Gadd (2000). TBT is also involved in growth inhibition, perturbation of the respiratory chain and membrane physical properties as evidenced by Martins *et al*. (2005) in two *Bacillus* sp. strains. In *E. coli* (Singh & Singh, 1985) and *Legionella pneumophila* (Soracco & Pope, 1983), TBT causes inhibition of ATPase, oxidation of substrates, and have effects in glycolysis and solute transport (Cooney and Wuertz (1989).

Table I - Microbial processes affected by TBT. Adapted from Gadd (2000)

Process affected	Organism/organelle
Respiration	Bacteria
Photosynthesis	Cyanobacteria
Nitrogen fixation	*Anabaena cylindrical*
Primary productivity	Microalgae
Growth	Microalgae
Energy-linked reactions	*E. coli*
Growth	*Aureobasidium pullulans*
Growth/metabolism	Fungi
Growth/metabolism	Bacteria
Photophosphorylation and ATP synthesis	Chloroplasts
H^+-ATPase activity	*Neurospora crassa*

3.1. Resistance to TBT

It is known and well reported that some bacteria are resistant to different TBT concentration levels (Dubey & Roy, 2003; Gadd, 2000). Presently, the resistance mechanism is not clearly understood, but it is known that resistant bacteria can: i) tolerate TBT due to inherent capability to transform it into less toxic compounds, such as dibutyltin (DBT) and monobutyltin (MBT) by a dealkylation mechanism (Table II) (Pain & Cooney, 1998); ii) to exclude TBT from the cell mediated by a multidrug efflux

pump (Jude *et al*, 2004); iii) can be used as a carbon source (Cruz *et al*, 2007; Pain & Cooney, 1998); iv) somehow can be bioaccumulated into the cell without breakdown the compound (Blair et al, 1982; Fukagawa et al, 1994)

Mendo *et al*. (2003) have demonstrated that the susceptibility to TBT varies in bacteria according to the structure of the cell wall. It was demonstrated that TBT is less toxic to Gram negative bacteria, where growth is observed up to 900 ng Sn ml^{-1}, then to Gram positive bacteria where, growth suppression occurs over 400 ng Sn ml^{-1} (Mendo et al, 2003). As reviewed by Dubey and Roy (2003) among some of the listed Gram negative bacteria resistant to organotin are *E. coli*, *Pseudomonas fluorences*, *P. aeruginosa*, *Proteus mirabilis*, *Serratia marcescens* and *Alicaligenes faecalis*, and the Gram positive *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Mycobacterium phlei* and *Vibrio* spp.

4. TBT degradation

Several studies suggest that TBT can be degraded into less toxic compounds, influenced by abiotic and biotic factors. TBT may, under favorable conditions, be degraded by successive dealkylation to produce DBT and MBT and finally inorganic tin, as reported by Dubey and Roy (2003) (Table II).

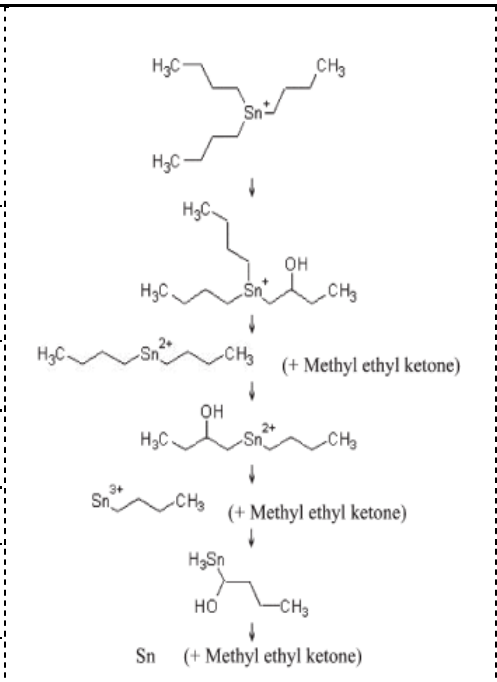
Abiotic factors influencing TBT degradation include solubility, dissolved/suspended organic matter, pH, salinity, temperature and light which may affect the availability of the compound to the microorganism, thus influencing TBT toxicity (Dubey & Roy, 2003).

There are some reports that demonstrated that TBT can be biodegraded by bacteria, fungi, cyanobacteria and seaweed in terrestrial and aquatic environment. Nevertheless the mechanism of biodegradation is not clearly understood and there is still a need to clarify the physiologic process, the levels of tolerance, the influence of anionic radicals and the importance of microbes in natural habitats (Antizar-Ladislao, 2008; Dubey & Roy, 2003; Gadd, 2000). Dowson *et al.* (1996) demonstrated that biotic

factors are the most important on TBT degradation in freshwater and estuarine sediments.

A recent work carried by Sakultantimetha *et al.* (2009) identified two new TBT resistant bacteria capable of biodegrading the compound. One of the isolates, *Enterobacter cloacae*, was then employed on bioremediation studies (Sakultantimetha *et al*, 2010a; Sakultantimetha *et al*, 2010b).

Table II – Degradation of TBT via successive dealkylation. Adapted from Antizar-Ladislao (2008)

Compound	Chemical structure	Enzyme	Formula
Tributyltin, TBT		TBT dioxygenase	$C_{12}H_{27}Sn^+$
β-hydroxybutyl-dibutyltin		DBT dioxygenase	$C_{12}H_{27}OSn^+$
Dibutyltin, DBT			$C_8H_{18}Sn^+$
β-hydroxybutyl-butyltin		MBT dioxygenase	$C_{18}H_{18}OSn^+$
Monobutyltin, MBT			$C_4H_9Sn^+$
β-hydroxybutyl			$C_4H_{12}OSn^+$
Tin			Sn^{4+}

5. Bioremediation of contaminated sites/environment

Some microorganisms are able to degrade or accumulate a variety of organic compounds that exists as contaminants in the environment. This is the fundament for bioremediation, where the metabolic potentials of microorganisms are explored/enhanced in order to optimize the decontamination of a given environment (Megharaj *et al*; Perelo, 2010).

The major benefit of bioremediation is the low cost relatively to physicochemical strategies, and in addition, it is a non-invasive and permanent technique leaving the ecosystem intact. Bioremediation also presents advantage when applied at contaminated sites with low toxic concentration, which would be impracticable when conventional remediation techniques are applied. Although, bioremediation has some drawbacks that can limit its application, as for instance, taking longer and being less predictable than conventional methods (Perelo, 2010).

Strategies for bioremediation include: (i) monitored natural recovery (MNR): some places have natural occurring chemical and physical process which associated with the microflora biodegradation leads to “self-remediation”; (ii) biostimulation: addition of key nutrients and manipulation of abiotic factors (e.g.: pH, temperature) that will enhance the biodegradation of the contaminants; (iii) bioaugmentation: introduction of appropriate species that show skills to degrade the contaminant; (iv) phytoremediation: use of plants or algae in the degradation and/or removal of contaminants from the environment (Megharaj et al; Perelo, 2010).

5.1. Use of microorganisms for bioremediation

The development of genetically engineered microorganisms (GEM) is an important tool to develop new strategies for remediation and monitoring of contaminated sites. Modification of enzymes, pathway construction and regulation, bioprocess development, monitoring and control, and biosensor development are the major approaches for the application of GEM in bioremediation. In table III are shown

some examples of GEM that have great degradation capacity and some that have been applied in bioremediation (Menn *et al*, 2008).

It is of great importance to monitor contaminated environments, either by determining the content/concentration of the toxic compounds or to assess the modifications of microbial populations. Molecular biology had brought new insights into bioremediation, mainly in bioaugumentation since it allowed the identification and characterization of the microbial population without cultivation, important when exogenous strains are added, given more consistent results in monitoring (Sayler & Ripp, 2000; Watanabe, 2001).

Biosensors to monitor contaminated sites are being adopted as suitable alternatives or complementary analytical tools in current days. As this emerging technology offers great advantages when compared with the conventional analytical tools, biosensors are able to identify and quantify specific compounds, with high sensitivity and accuracy (Durand *et al*, 2003; Rodriguez-Mozaz *et al*, 2005). Nevertheless research is still needed to improve them. It is in this wide aim that the present work is focused. *A. molluscorum* Av27 was reported to be highly resistant to TBT and also has having the ability to degrade it (Cruz *et al*, 2007). Thus Av27 can be potentially improved as a tool for TBT decontamination (Cruz *et al*, 2010) and therefore, studies are in progress to achieve this aim. Hence, it is of major importance to understand all the pathways that are involved in the TBT resistance/degradation ability exhibited by this strain.

Table III – GEM applied in biodegradation of contaminants and in biodegradation process efficacy. BTEX - benzene, toluene, ethylbenzene, and xylene; 2,4-D - 2,4-dichlorophenoxyacetic acid; PBC - polychlorinated biphenyls; TCE – trichloroethylene. Adapted from Menn et al. (2008)

Microorganism	Modification/Application	Contaminant
***Pseudomonas* sp. B13**	pathway	mono/dichlorobenzoates
P. putida	pathway	4-ethylbenzoate
***P. putida* KT2442**	pathway	toluene/benzoate
***Pseudomonas* sp. FR1**	pathway	chloro-, methylbenzoates
***C. testosteroni* VP44**	substrate specificity	*o*-, *p*-monochlorobiphenyls
***Pseudomonas* sp. LB400**	substrate specificity	PCB
***E. coli* JM109 (pSHF1003)**	substrate specificity	PCB, benzene, toluene
***P. pseudoalcaligenes* KF707-D2**	substrate specificity	TCE, toluene, benzene
***E. coli* FM5/pKY287**	regulation	TCE, toluene
***A. eutrophus* H850Lr**	process monitoring	PCB
***P. putida* TVA8**	process monitoring	TCE, BTEX
***P. fluorescens* HK44**	process monitoring	naphthalene, anthracene, phenanthrene
***B. cepacia* BRI6001L**	strain monitoring	2,4-D
***P. fluorescens* 10586s/pUCD607**	stress response	BTEX
***P. fluorescens* 10586s/pUCD607**	toxicity assessment	chlorobenzenes, chlorophenols, BTEX
***Pseudomonas* strain Shk1**	toxicity assessment	Cd, 2,4-dinitrophenol, hydroquinone
***A. eutrophus* 2050**	end point analysis	nonpolar narcotics

6. A multidrug transporter family - Small multidrug resistance proteins

Small multidrug resistance proteins (SMR) comprehend a family of small integral inner membrane proteins, with approximately 12kDa and 100 to 140 amino acids in length (Paulsen *et al*, 1996b). The SMR protein family belongs to the drug/metabolite transporter (DMT) superfamily (Jack *et al*, 2001; Putman *et al*, 2000; Saier & Paulsen, 2001; Saier, 2000).

SMR proteins family confers resistance and has only been reported to transport quaternary ammonium compounds (QAC), other lipophilic cations and may also transport a variety of antibiotics (Bay *et al*, 2008; Heir *et al*, 1999; Jack *et al*, 2000). Its structure is characterized by four transmembrane (TM) α-helices domains with short hydrophilic loops, turning SMR highly hydrophobic, making them solubilized in organic solvents (Yerushalmi *et al*, 1995; Yerushalmi *et al*, 1996). It has also been reported that the transport is mediated via electrochemical proton gradient force (Paulsen *et al*, 1996a; Paulsen *et al*, 1996b; Yerushalmi *et al*, 1995), and therefore classified as proton-dependent multidrug efflux system (Paulsen *et al*, 1996a).

Once not all the proteins in the SMR family demonstrate drug efflux, in the beginning, the SMR family was divided into two distinct classes: i) small multidrug pumps (SMP) and ii) suppressor of groEL mutation proteins (SUG). This division was supported by the phenotype that they confer (Greener *et al*, 1993; Saier *et al*, 1998) and by the phylogenetic assessments (Chung & Saier Jr., 2001; Paulsen *et al*, 1996b).

Multidrug efflux pumps were identified and characterized, and seem to have homology with SMR proteins. These homologues are distinct from the other two groups of SMR proteins, once it is necessary the co-expression of two separate SMR genes within the host to demonstrate the same resistance profile of SMP and SUG (Chung & Saier Jr., 2001). An example of this paired SMR proteins are *Bacillus subtilis* EbrA and EbrB (Masaoka et al, 2000), YkkC and YkkD (Jack *et al*, 2000). Also, Bay *et al*. (2008) proposed that this family is divided in three subclasses: the small multidrug protein (SMP), the suppressor of groEL mutation protein (SUG) and paired SMR (PSMR).

6.1. SMP subclass

The ethidium multidrug resistance protein (EmrE) from *Escherichia coli* is considered the representative model of the SMR family, and belongs to the SMP subclass, being the best known and well characterized protein from this family (Schuldiner *et al*, 2001). Previously named as *mvr*C, the *emr*E gene was first identified and cloned from the genome of *E. coli*, regarding its resistance to ethidium bromide (Purewal, 1991) and to methyl viologen (Morimyo *et al*, 1992).

It has been demonstrated that two types of residues in multidrug resistance proteins are involved in binding to QAC. A negatively charged residue binds to the positive charge of the ligand, highly conserved in the multidrug resistant proteins (Muth & Schuldiner, 2000; Paulsen *et al*, 1996b). In EmrE, a glutamate residue located in the first TM helix is present for this purpose (Muth & Schuldiner, 2000). Also aromatic residues are important in the protein-drug interaction; they are involved in Van der Waals forces and π-π interactions with the aromatic rings of the ligand (Dougherty, 1996; Zhong *et al*, 1998). EmrE contain the residues Y40, Y53, F44 and Y60 and W63 that may play a role in the interaction protein/ligand. Reports demonstrated that when some of these residues are mutated in EmrE, the protein is non-functional (Elbaz *et al*, 2005; Mordoch *et al*, 1999; Yerushalmi & Schuldiner, 2000a).

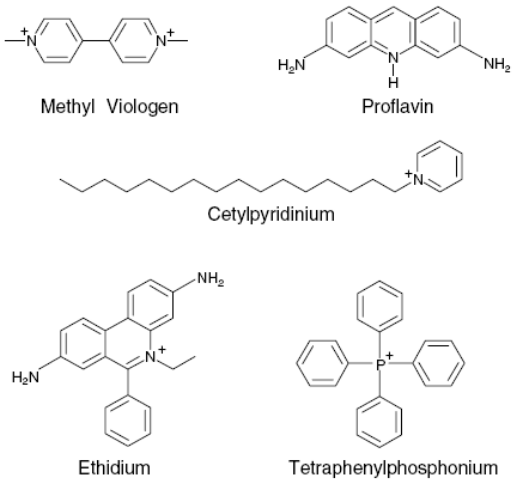


Fig. 3 - Lipophilic cations used in the Sikora and Turner (2005a) experiments.

Sikora and Turner (2005a) used isothermal titration calorimetry (ITC) to monitor the binding of some lipophilic cations (fig. 3) to EmrE in different membrane mimetic environments, thus providing information about the stoichiometry and thermodynamic properties of protein-ligand interactions. The binding stoichiometry of EmrE to drug was found to be 1:1 (mol/mol), indicating that oligomerization of EmrE is

not necessary for binding the drug. Nevertheless, the oligomerization is necessary for the transport of the drug across the cell membrane, once another EmrE subunit is required to bind protons necessary for the transport mediated via electrochemical proton gradient force (Muth & Schuldiner, 2000; Yerushalmi & Schuldiner, 2000a; Yerushalmi & Schuldiner, 2000b). Indeed, Bay *et al*. (2010) showed that the multimeric forms of EmrE are influenced by the concentration of SDS and it is stabilized by the ligand in the mimetic environment, demonstrating that EmrE is capable of multimeric flexibility, altering its subunit amount to correspond to ligands.

6.2. SUG subclass

One of the genes that seem to be involved in the TBT resistance by Av27, is a homologue of the *sug*E gene, and encode a protein designated SugE-Av27. Thus this last is probably a member of the SUG subclass of the SMR family.

SUG subclass is poorly studied and understood. SugE was firstly described and identified as a suppressor of a groEL mutation and in addition it could mimic the effect of GroEL over-expression in a *Klebsiella pneumoniae* (Greener *et al*, 1993). GroEL, homologous to Hsp60 (eukaryotic heat shock protein), is part of the GroEL/GroES chaperone complex within bacteria. Chaperones helps in the proper folding of proteins in natural conditions, as also in cellular damage by heat shock or others stress conditions (Georgopoulos, 2006). Afterwards SugE was included in the SMR family since its sequence showed high homology with others SMR proteins, like EmrE (Bay *et al*, 2008; Chung & Saier Jr., 2001). In *E. coli*, SugE and EmrE share 27% sequence identity and 52% sequence similarity (Sikora & Turner, 2005b). However, SugE is not capable to recognize or transport the diverse QAC and lipophilic dyes demonstrated by SMP proteins (Chung & Saier, 2002). SugE confers resistance phenotype to a narrow range of compounds, mostly to antiseptics, with acyl chains covalently bound to a single N cation, like cetylpyridinium, cetyldimethylethyl ammonium and hexadecyltrimethyl ammonium (Chung & Saier, 2002).

Mutagenesis assays carried by Son *et al*. (2003) showed that when key residues of SugE, equivalent to those preserved in the SMP subclass, were mutated (fig. 4), the clones would became hypersensitive to all drugs tested. This report shows that SugE is functioning as a drug importer (Son *et al*, 2003). The orientation in the membrane of both SugE and EmrE was determined to be N- and C- terminal in the cytoplasm side of the inner membrane; this exclude that the orientation of SugE was the cause of the importer activity (Son *et al*, 2003). When the histidine-24 (conserved in the SUGs) is exchanged for glutamate (conserved in the SMPs), there is an exchange from a positively charged residue to a negatively charged residue, leading to a hypersensitivity to the more positively charged compounds (Son *et al*, 2003). In the SMP protein QacC from *Staphylococcus aureus*, the mutation of Glu-24 demonstrated that this residues is involved in determining the specificity of drug resistance (Grinius & Goldberg, 1994). These evidences and another experiments with EmrE (Edgar & Bibi, 1997; Grinius & Goldberg, 1994; Heldwein & Brennan, 2001; Muth & Schuldiner, 2000; Paulsen *et al*, 1996b) reveals that these conserved residues in SugE and EmrE are involved in drug binding to the protein.

Sikora and Turner (2005b) have demonstrated that SugE and EmrE have similar affinity and stoichiometry to drugs. Like EmrE (Sikora & Turner, 2005a), SugE shows to bind to drugs in a ratio 1:1 (one drug bind to one SugE subunit) with similar strength (Sikora & Turner, 2005b). Sikora and Turner (2005a; 2005b) have postulated too, that the drugs initially has a weak binding into the membrane, and therefore, once it is energetically favorable, the drug enters into the binding pocket of SugE.

The conformation of SugE has never been confirmed and explored, till a recent study carried by Bay and Turner (2011). It was reported that SugE protein appears like a monomer in Tricine SDS-PAGE, when independently solubilized in two detergents, sodium dodecyl sulphate (SDS) and dodecyl maltoside (DDM). However, SugE appears as a dimer at higher protein concentration in SDS (Bay & Turner, 2011).

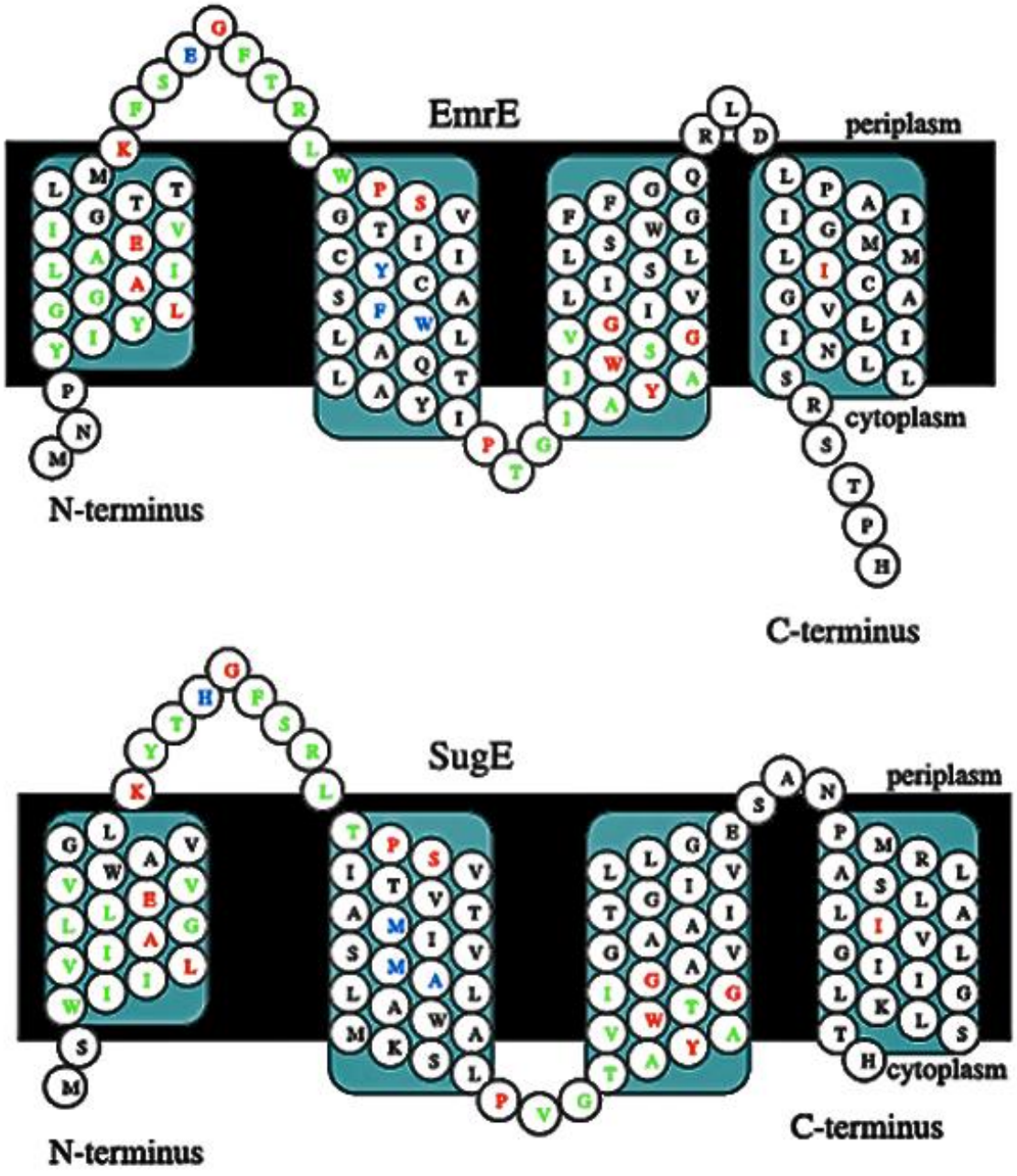


Fig. 4 - Schematic representation of *E. coli* SugE and EmrE showing their sequence and topology. The residues in blue are the ones that were mutated to convert a SugE into an EmrE. Residues that are conserved in all members of the SMR family are in red. The green residues are the SMR similar motifs (Son et al, 2003).

Contrary to what is observed with EmrE, multimerization of SugE didn't seem to be influenced by increasing QAC : protein molar ratio, although, the electrophoretic mobility is altered (Bay & Turner, 2011).

It was demonstrated that EmrE and SugE proteins are quite similar. Both have transmembrane α-helix content, the tertiary conformation can be altered by the environment (ratio of drug:protein, detergent) and also SugE and EmrE have conserved residues at similar positions, that are responsible for recognizing and binding the drug with similar stoichiometry and strength. Only the events that take place after binding are different; just EmrE shows resistance to all drugs tested (fig. 4) (Sikora & Turner, 2005b). Further studies are still needed to better clarify the function and the mode of action of these SUG proteins.

7. Methodological approaches

TBT resistance by Av27 strain is known to be linked to the relative over-expression of the *sug*E-Av27 gene (Cruz *et al*, 2010). SugE protein belongs to the SUG subclass of the SMR proteins family and little is known about this subclass (Bay et al, 2008). Thus it is crucial to understand how the SugE-Av27 protein works and how is it involved in the TBT resistance in Av27. To that end, purification of this protein is needed.

Not all the proteins are naturally present in an aqueous phase; actually, in prokaryotes, there are cell membrane proteins and in Gram-negative bacteria, periplasmic proteins that are partly immobilized between the outer and inner membranes of the cell. For their isolation and purification special needs must be considered and in each case analyzed individually (Scopes, 1993).

The membrane proteins can be divided in two different types, peripheral or integral. If we are dealing with a peripheral membrane protein, they can be easily isolated by easygoing treatments that do not involve solubilization of the membrane. On the other hand, with integral membrane proteins special conditions are needed,

once they are embedded within and often right across the membrane, which isolation and purification will almost always need a solubilization step of the membrane (Scopes, 1993).

From the previous studies of the gene *sug*E-Av27, and the predicted aa sequence of the protein, it is expected that the SugE-Av27 protein is an integral membrane protein from the inner membrane of Av27 strain (data not published). For the isolation of this protein isolation and solubilization of the inner membrane is needed. The addition of the Triton X-114 detergent to the extraction protocol, as the one described by Arnold and Linke (2008), will help to achieve this purpose (fig. 5).

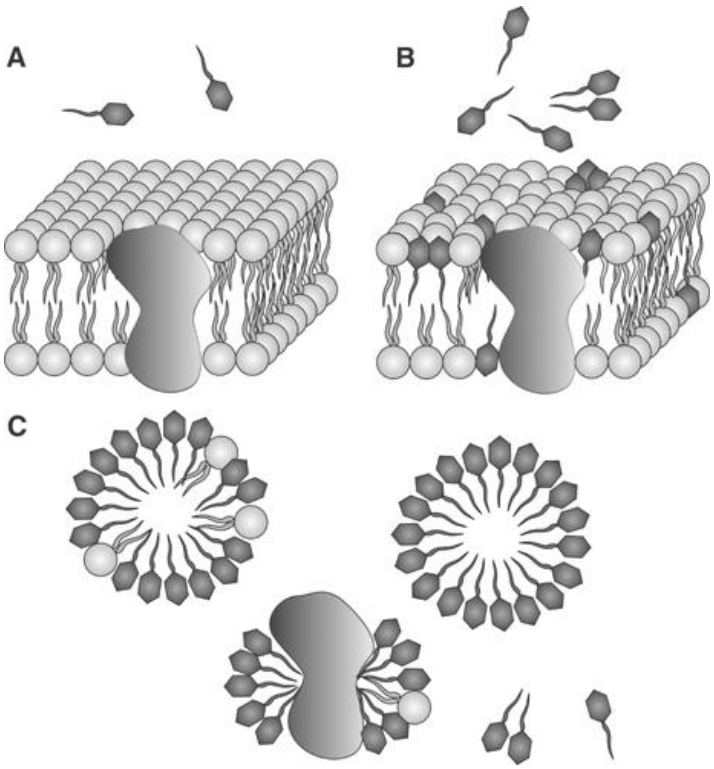


Fig. 5 - Mode of action of the detergent on biomembranes. Detergents insert into the bilayer (A and B), and at high concentrations form mixed micelles with lipids and membrane proteins, keeping the proteins soluble (C) (Arnold & Linke, 2008).

In the present work, mass spectrometry analysis was the technique selected, to detect and identify SugE-Av27 protein in the membrane protein extract of Av27 strain. Mass spectrometry is an analytic technique that measures the masses of individual molecules, often referred as analytes, which are first ionized and separated according to its mass-to-charge ratios (m/z). The protein sample is digested into smaller peptides, which are characterized by mass measurements or sequence ions analysis. Using the appropriate software, comparing to proteins from the database, a probable identification is obtained (Dass, 2001; Westermeier & Naven, 2002).

The mass spectrometer can be divided into three different parts, which involve the three basic steps in mass spectrometry:

- the ion source: where the molecular ions are produced. Ionization converts the analyte molecules into gas-phase ionic species, by the removal or addition of electrons or protons;
- the analyzer: where the molecular ions and their charged fragments are separated and analyzed on the basis of their m/z ratios;
- the detector: where ions are detected. The mass-separated ions are measured, amplified and displayed through software in the form of a mass spectrum.

MALDI-TOF is a well established mass spectrometry technique which has proved its abilities for identifying proteins, peptides and some other ionisable compounds in samples. The association of MALDI source to a TOF analyzer (fig. 6) enables the analysis of larger biomolecules avoiding its breakdown and degradation by the temperature elevation triggered by laser incidence (Constans, 2005).

Fig. 6 - MALDI-TOF/TOF analyser

In order to characterize a protein, it is necessary to get it pure and in high amounts. Purification of the protein directly from the host strain could be difficult; also obtaining high concentrations of the protein might also be difficult. Since normally there are only a few molecules per cell. Thus, recombinant DNA technology seems to be the solution to overcome this problem (Maloy et al, 1994).

Heterologous expression in a host strain such as *E. coli*, with the selected expression vector pET24 (fig. 7), could allow the production of the target protein with a histidine-tag (his-tag). The protein, SugE-Av27_his-tag, can be easily detected with specific antibody anti-his-tag in a Western blot analysis. Also, it is possible to determine the best conditions for maximum expression of the protein. Furthermore, the purification of the protein will be easily achieved by immobilized metal ion affinity chromatography (IMAC) with Ni^{+} that has high affinity to his-tag (Terpe, 2003).

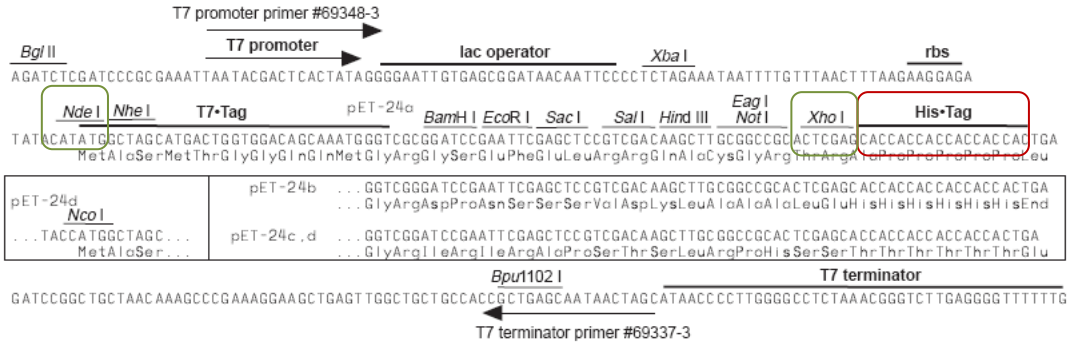


Fig. 7 – pET24 vector cloning/expression region (NOVAGEN). Red square indicates the region in the vector which encodes the his-tag; green squares indicates the restriction sites where was inserted the *sug*E-Av27 gene; rbs - ribosome binding site.

Gas chromatography - mass spectrometry (GC-MS) is one of the analytic techniques that is normally employed to analyze the content of organotin compounds in environmental and laboratorial samples (Antizar-Ladislao, 2008). This technique is expensive and time consuming. Optimization of one simple and rapid method to detect the degradation/content of TBT/organotins prior to send samples for analysis, in laboratorial assays will be a time/cost benefit to the development of a study.

Micrococcus luteus is known to be an indicator specie in many toxicological assays (Caetano, 2011). In the present work, the use of this indicator specie to indicate TBT degradation will be evaluated.

B. Objectives

TBT contamination in the environment is a worldwide problem. Screening for microorganisms resistant to this compound allowed the isolation of *Aeromonas molluscorum* Av27, a Gram negative bacterium highly resistant to TBT that shows the ability to degrade it into its less toxic products: DBT and MBT. Genomic studies with Av27 strain revealed the presence of the gene *sug*E-Av27 that encodes an inner membrane protein involved in the resistance mechanism. This protein belongs to the SMR protein family. Understanding how this protein is involved in the resistance to TBT and also evaluating the TBT degradation capacity by Av27 are the main goals of this work.

With that purpose the following aims were addressed:

i) Verify the over-expression of SugE-Av27 protein in Av27 in the presence of TBT ;
ii) Clone *sug*E-Av27 gene into an *E. coli* expression system, which will allow further purification of the protein for future characterization studies;
iii) Development and optimization of a simple and rapid method to evaluate the TBT degradation/toxicity.

C. Material and Methods

1. Extraction of SugE protein from Av27

1.1. Protocol 1

Av27 was grown at 26°C at 160rpm in a 500ml sterile Erlenmeyer flask, in two conditions: (i) 100ml of TSB medium (MERCK) without TBT; and (ii) 100ml TSB medium (MERCK) containing 500µM TBT; both conditions were made in dark to avoid photochemical degradation of TBT. Once achieved an $O.D._{600\ nm} \approx 0.2$, the culture was harvested by centrifugation at 4000g for 10min and the cellular pellet was stored at -20°C.

Extraction of membrane proteins was achieved with an adaptation of the protocol from Arnold and Linke (2008). Cellular pellet was resuspended in 500µL of 1x phosphate buffered saline (PBS) (VWR International Ltd.). The suspension was centrifuged at 13000 g for 1 min (this two first steps were repeated twice). The pellet obtained was then solubilized in 400 µL of solubilization solution (10mM Tris-HCl pH 7.4, 150mM NaCl, 1% (v/v) Triton X-114), incubated 10min at 4°C and centrifuged at 12000g for 30s at 4°C. To the solubilized culture (top), a sucrose solution (10mM Tris-HCl pH 7.4, 150mM NaCl, 0.06% (v/v) Triton X-114, 6% (w/v) sucrose) was added carefully and incubated for 10min at 37°C. A centrifuging step at 12000g for 5min at 4°C separated the solution in two phases. The aqueous phase (top) and detergent phase ("oil" in the bottom) was recovered into a new and clean eppendorf. Triton X-114 was added to the aqueous phase at a final concentration of 2% (v/v) and the separation step was repeated. 100µL of 10mM Tris-HCl pH 7.4, 150mM NaCl Buffer was added to the detergent phase. Finally 10 volumes of ice-cold acetone (-20°C) was added to each final samples and leaved overnight at 4°C. To recover the precipitated protein was centrifuged at 12000g for 20min at 4°C. The pellet was then resuspended in 100µL of 10mM Tris-HCl pH 7.4, 150mM NaCl Buffer.

1.2. Protocol 2

Since with the previous protocol, it was not possible to detect the SugE-Av27 protein by MS analysis (see results and discussion section), another protocol was attempted that consisted in an adaptation of the one from Winstone et al. (2002).

Av27 cells were grown at 26°C at 160rpm in a 5L sterile Erlenmeyer flask, in two conditions: (i) 1L of TSB medium (MERCK) without TBT and (ii) 1L of TSB medium (MERCK) containing 500µM TBT; both conditions were made in dark to avoid photochemical degradation of TBT. Once achieved an $O.D._{600\ nm} \approx 0.2$, cultures were harvested by centrifugation at 4000g for 10min and the cellular pellet was stored at -20°C.

Frozen Av27 cell pellets were thawed at 4°C and resuspended in 2ml of SMR A buffer (50mM MOPS, 5mM EDTA, 1mM DTT, 8% v/v glycerol, pH=7.5) per gram of cell mass. 1µL of 10mM phenylmethanesulfonylfluoride (PMSF) was added per milliliter of cell suspension immediately prior to sonication (10s, 5x, spaced by 10s). After sonication the low speed pellet (LSP) or unbroken cell debris was collected by centrifugation at 9000g for 15min and the supernatant was centrifuged at 110,000g for 1h30min to collect the membrane pellet. The supernatant (cytosol) fraction was removed and the membrane pellet was resuspended in SMR A buffer till complete solubilization. This membrane suspension was then frozen in aliquots and stored at -80°C prior to further processing

1.3. Determination of protein concentration

Protein concentration of the extracts was determined using the Qubit™ Protein Assay Kits from Invitrogen.

1.4. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed on a Bio-Rad Mini-PROTEAN Tetra Cell apparatus using a 6% acrylamide stacking gel, 18% and 20% acrylamide separating gel (Appendix 1). Protein samples were solubilized in SDS–PAGE loading buffer (125 mM Tris, 4% w/v SDS, 20% w/v glycerol, 0.0025% w/v bromophenol blue and 2.5% beta-mercaptoethanol, pH 6.8) at proportions 1:1, for 5 min at 95°C prior for loading. Gels were stained with coomassie blue stain (0.1% w/v coomassie R250, 50% v/v metanol, 10% v/v acetic acid) overnight and de-stained with 15% v/v methanol, 10% v/v acetic acid for a minimum of 3h (Laemmli, 1970).

Tricine SDS-PAGE were prepared as described above with the exception that 16% acrylamide separating gels were made (Appendix 1). The anode buffer was made to contain 0.2M Tris, pH 8.9 while the cathode buffer containing 0.1M tricine in addition to 0.1M Tris and 0.1% w/v SDS, pH 8.25. Tricine SDS-PAGE samples were processed identically to SDS-PAGE samples and were also run, stained and destained identically to SDS-PAGE.

1.5. Mass spectrometry analysis

For the proteins identification, the bands presented in the SDS-PAGE were excised and were added 20µl of ammonia bicarbonate 10% and left to incubate for 30 min, then added 20µl of acetonitrile (ACN) and left for more 30min. The bands were decanted and the two previous steps repeated twice (when performed a Tricine SDS-PAGE the steps were repeated 4 times). The samples were then evaporated on a SpeedVac. Once dried, 25µl of 10µg/ml trypsin in 50mM ammonium bicarbonate was added to the sample and left at 37°C overnight. The tryptic peptides were extracted from the gel with formic acid and were then dried in vacuum and resuspended in 10µl of a 50% acetonitrile/0.1% formic acid solution.

The full extracts (protocol 1 and 2) were incubated with trypsin and the tryptic digests were separated using an Ultimate 3000 (Dionex/LC Packings, Sunnyvale, CA). Ten microlitres of each sample (corresponding to 1µg of protein) were injected onto a C18 trapping column (Pepmap300, 5um particle size, 5mm, Agilent Technologies) using an autosampler (Dionex/LC Packings). The sample was washed over the trapping column for 5 min with 95% buffer A (water, 0.1% TFA), 5% buffer B (ACN, 0.05% TFA) at a flow rate of 300 nl/min. Afterwards, the sample was eluted onto a 150 mm x 75 µm PepMap100 capillary analytical C18 column with 3mm particle size (Dionex/LC Packings) at a flow rate of 300 nl/min. A linear gradient of 5–50% buffer B was run over a period of 35min. The separation was monitored at 214nm using a UV detector (Dionex/LC Packings) equipped with a 3nl flow cell. The peptides eluting off the C18 capillary column were directly mixed with α-CHCA matrix solution (2mg/ml in 70% ACN/0.3% TFA) containing internal standard Glu-Fib (15 ftmol for MALDI-TOF/TOF MS analysis) under a continuous flow rate of 270nl/min. The fractions were then deposited onto the LC-MALDI plates at 20s intervals for each spot (100 nl/fraction), starting 5min after the beginning of the separation process, using the Probot (Dionex/LC Packings).

The MALDI-TOF/TOF MS analysis of the samples was performed using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). Screening of the LC-MALDI plate was performed in the MS positive reflector mode using 1200 laser shots and TOF-TOF MS analysis of automatically selected precursors was performed at a collision energy of 2kV using air as collision gas at a pressure of 2 x 10^{-7}torr. MS spectra were internally calibrated using Glu-Fib and several trypsin autolysis products. Up to ten of the most intense ion signals per spot position with S/N ratio above 50 were selected as precursors for MS/MS.

The spectra were processed and analysed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v.2.1.0.4, Matrix Science, UK) for protein/peptide identification based on the peptide mass fingerprints and MS/MS data. The search was performed against the Swiss-Prot protein database. MS tolerance of 30ppm for precursor ions and 0.3Da for fragment

ions, two missed cleavages and carboxymethylation of cysteines were selected for protein identification. Protein identification was accepted as positive when both the MASCOT total ion scores for a given protein and its best peptide individual score exhibit confidence intervals higher than 97%.

2. Expression of SugE protein in *Escherichia coli*

To get full amounts of SugE-Av27 protein to fully characterization, it was done heterologous expression in *E. coli* system with his-tag for forward purification with IMAC technique.

Three different clones were made, one with the *sug*E-Av27 gene attached to the his-tag (clone BL21_sugE_HT), one with the *sug*E-Av27 gene without a tag (clone BL21_sugE_STOP) and another one only with the original vector (clone BL21_pET24). These two last ones were made for controls in the induction and purification protocols.

2.1. Amplification of the target gene

Specific primers were designed to amplify the target gene and containing, at each terminal, the NdeI or XhoI restriction sites (Appendix 2), allowing ligation into the NdeI/XhoI pET24 vector (fig. 7, Introduction section). The PCR amplification was made with Thermo Scientific Taq and buffers. The PCR reaction protocol was 50µL of Master Mix, 3µL of forward primer and reverse primer, 42µl of dH_2O and finally 2µL of Av27's cells suspension. The PCR protocol and program can be seen in the Appendix 2. After the amplification, the PCR product was purified with the Jetquick PCR Product Purification kit from GENOMED.

2.2. Digestion and ligation of pET24 vector and PCR product

The vector pET24a(+) (NOVAGEN) was extracted from the *E. coli* DH5α_pET24 by QIAprep® Spin Miniprep Kit (QIAGEN). The pET24 and PCR products digestions were made with the restriction enzymes NdeI, XhoI and respective buffer from FERMENTAS. The reaction mixture can be viewed at the table IV; the incubation was made at 37°C for 3h.

Table IV – Reaction mixture for the digestion of the pET24 vector and the PCR product.

	pET24	**PCR product**
DNA	20 µL	10 µL
XhoI (10 U/µl)	4 µL	4 µL
NdeI (10 U/µl)	2 µL	2 µL
Orange buffer (10X)	4 µL	4 µL
dH_2O	10 µL	20 µL

Once the digestion was completed the digested inserts and plasmid were purified with the Jetquick PCR Product Purification kit from GENOMED. The concentration of DNA was measured using the Qubit™ dsDNA HS Assay Kits.

For the ligation of each insert to the pET24 vector was made independently the following reaction: 1 µL of pDNA (52 ng), 3 µL of Insert (84 ng), 2 µL Buffer (10x), 13 µL of d H_2O and 1 µL of T4 DNA ligase enzyme (5 U/µl) from FERMENTAS. The reaction mixture was incubated at room temperature (± 24°C) for 1h.

2.3. Preparation of competent *E. coli* BL21 cells

E. coli BL21 (DE3) cells glycerol was stored at -80°C. A pre-inoculum was made with an aliquot from the glycerol, and then 1ml added to 50ml LB medium containing 15µg/ml tetracycline and incubated till a DO_{600} ≈ 0.4-0.6. The cell suspension was centrifuged at 7000g for 5min. The pellet was washed with 25ml of $MgCl_2$, and once again, centrifuged and washed with 25ml of $CaCl_2$ letting to incubate for 20min at 4°C. The washed cells were centrifuged at 7000g for 1min and the pellet resuspended in 1.5 ml $CaCl_2$, glycerol buffer. The full competent cells were divided in aliquots of 50 µl and stored at - 80°C.

2.4. Transformation of competent *E. coli* BL21

For the transformation, 5µl of each pDNA (pET24_sugE_HT, pET24_sugE_STOP and pET24) independently, was added to 50µl of BL21 competent cells and left on ice for 15min, then transferred to 42°C dry bath for 45 sec and right away again in ice for 2min. Finally, added 1ml of LB medium without antibiotic and incubated at 37°C 160rpm for 1h. The cell suspension was centrifuged at 5000g for 1min and the pellet decanted and resuspended in the remaining medium. Cells were then plated in LB plates containing 50µg/ml of kanamycin (Kan) and left at 37°C overnight.

2.5. Screening of positive clones

For the selection of positives clones, 10 colonies of each transformation were randomly selected and analysed by colony PCR. The program and primers are listed in table 2.2 (Appendix 2).

To verify correct constructions of the plasmid in selected positive clones, colony PCR was performed with the appropriate primers T7prom and T7ter (Appendix 2). The PCR products were sent to STABVIDA for nucleotide sequencing.

2.6. Growth and induction of BL21 clones with IPTG

A single colony of each clone was selected and inoculated in 1ml LB containing 50µg/ml Kan, then incubated with shaking at 37°C until an OD_{600} ≈ 0.6. The cultures were stored at 4°C overnight. In the following morning, the cells were collected by centrifugation and resuspended in 1.5ml of fresh medium containing antibiotic. Incubation was made at 37°C with shaking at 250rpm till an OD_{600} ≈ 0.5. These 1.5ml cultures were used to inoculate independently 50ml LB with Kan in 250ml Erlenmeyer flasks. The cultures were incubated at 37°C with shaking at 250rpm until the OD was around 0.5-1.0. The 50ml cultures were divided into two 25ml cultures and 500µl IPTG (stock of 50mM) was added to one of the 25ml cultures and the other one was left as an uninduced control. The OD monitoring during growth was made by removing aliquots aseptically.

The induced and uninduced cultures were left at 37°C with shaking at 180rpm, 100µl samples were aseptically taken over 1, 2, 3, and 4 hours and the cells collected by centrifugation at 12000g for 1min. The cellular pellets were then stored at -20°C for prior analysis.

2.7. SDS-PAGE and Western blot analysis

The cell samples from each time and induction were prepared to SDS-PAGE like described in section 1.4 from Material and Methods.

For the Western blot, 3MM blotter paper was cut to fit the transfer cassette and a nitrocellulose membrane of the gel size was also cut. The gel was removed from the electrophoretic device and the stacking gel discarded. The transfer sandwich was assembled under transfer buffer (25mM tris, 192mM glycine, 10% methanol) to avoid air bubbles and placed in the transfer device of Bio-Rad Mini-PROTEAN Tetra Cell apparatus and filled with transfer buffer and left to transfer for 2h at 200mA. Once finished, the membrane was removed carefully and soaked in TBST (10mM Tris-HCl, 150mM NaCl, 0.05% Tween, pH 8.0); the membrane was then blocked in 5% milk in TBST for 1h.

ECL^{TM} is a light emitting non-radioactive method for the detection of immobilised antigens. The membrane was incubated with 5ml of the primary anti-body (his-tag monoclonal antibody from mouse, NOVAGEN) diluted (1:1000) in 3% low fat milk in TBST for 1h at room temperature and then overnight at 4°C. In the other day the membrane was decanted and washed three times with TBST for 10min. 5 ml of secondary antibody anti-mouse diluted (1:5000) in 3% low fat milk, was added and incubated for 2h at room temperature, with shaking. For the detection and revelation, all the next steps were made in a darkroom. The membrane was incubated for 1 min with 1ml of the ECL detection solution (a mixture of equal volumes of solution 1 and solution 2 from the ECL kit (Amersham), approximately 0.125ml/cm^2 membrane). Forward the membrane was wrapped in cling-film, taking care to eliminate all the air bubbles, and placed in a film cassette with an autoradiography film (XAR-5 film, KODAK) on top and exposed overnight. The film was developed in developing solution, washed in water and fixed in fixating solution.

3. Development of one simple and rapid method to monitor the TBT degradation/toxicity

3.1. Evaluation of *M. luteus* as an indicator specie for TBT degradation monitoring

Since *M. luteus* is sensible to a large variety of compounds, this microorganism is broadly used as indicator specie. Finding out about the sensibility of this microorganism to TBT and DBT, will open the possibility to use *M. luteus* as indicator specie, to evaluate the degradation of TBT by *A. molluscorum* Av27 in liquid assays.

The sensitivity of *M. luteus* to TBT and DBT was tested using different concentration of these compounds (25µM, 50µM, 100µM) in TSB plates (MERCK). Each assay was made in duplicate, one incubated at 37°C (optimal temperature for *M. luteus*) and another one divided in two parts, where Av27 and *M. luteus* were inoculated and incubated at 30°C (temperature that both microorganisms grow). In each case, control without TBT was made.

3.2. Optimization of the bioassay method to evaluate TBT degradation/toxicity

A liquid assay was made for the degradation of TBT experiment. Av27 was inoculated with 25µM of TBT in 100mL of Marine broth (MB) (DIFCO) and left incubating over time at 26°C, 150rpm. To monitor any potential photo- and chemical natural degradation of TBT, a control with 25µM TBT in 100mL of MB (DIFCO) was made and incubated under the same conditions. Aliquots of 100µL of these two conditions were taken and added to plates with incorporated *M. luteus*. It was incubated at 37°C for 24h. After that period, the presence or absence of one inhibition zone was evaluated. A 100µL aliquot of "fresh made" 25µM TBT in TSB plates as control of TBT effect on *M. luteus* was also applied in the plate. This assay was done on time 0, and after 6, 24, 30, 48 and 54h.

D. Results and Discussion

1. Membrane protein extraction from *Aeromonas molluscorum* Av27

An identical quantity of protein per samples from the protocol 1 (32.5 µg) and protocol 2 (30.1 µg) was separated by SDS-PAGE.

Analysis of the extracts from protocol 1, 15% SDS-PAGE showed weak resolution below the 20kDa zone (fig. 8). To solve this problem 20% SDS-PAGE were used instead, in which we could observe 13 bands with higher intensity in the TBT/Av27 sample when compared to 0/Av27 sample (fig. 8). These 13 bands were individually excised from the gel and analyzed by MALDI-TOF; the full extracts were also analyzed. When performing the SDS-PAGE with the samples recovered from protocol 2 the resolution of the gel was weak. Thus it was decided to use Tricine SDS-PAGE methodology, which allowed a better separation of the bands; from this gel, 8 bands were excised and analyzed by MALDI-TOF. The full extracts were also analyzed.

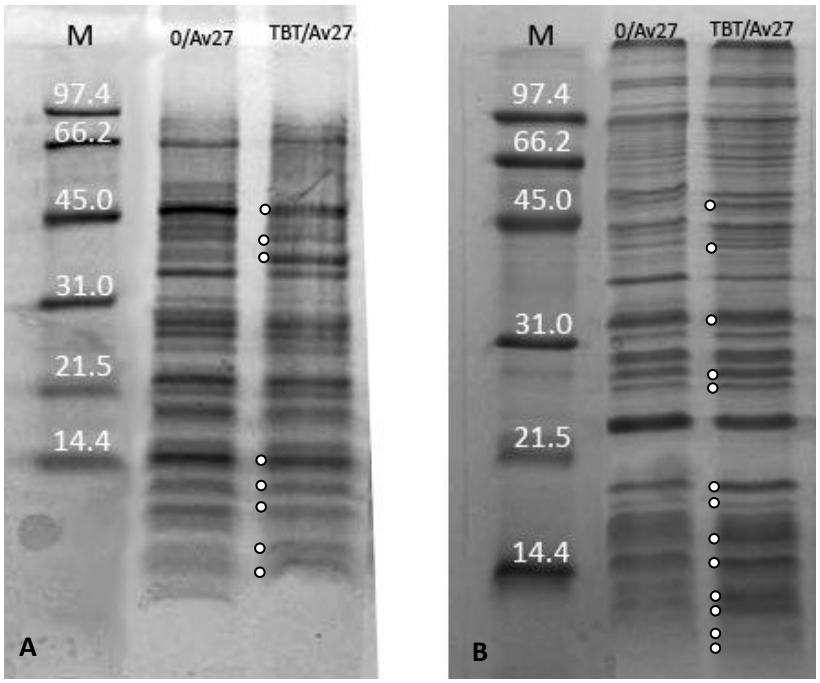


Fig. 8 - SDS-PAGE of the protein extracts from protocol 1 (B) and protocol 2 (A). White spots indicate bands excised (numerated bottom to top) for MS analysis. 0/Av27 - culture of Av27 without TBT; TBT/Av27 - culture of Av27 with 500 µM of TBT; M - SDS-PAGE MW standards, low range (Bio-Rad). A - 20% polyacrylamide gel; B – 16% Tris-Tricine polyacrylamide gel.

As can be seen in the table 3.2 (Appendix 3) with the SDS-PAGE and MS analysis it can be suggested that proteins associated with the protein synthesis (e.g.: ribosomal proteins, elongation factors), transcription of DNA (e.g.: RNA polymerase) and glucolysis (e.g.: pyruvate dehydrogenase) are being over-expressed. These results seem feasible, since the cell has to cope possibly with TBT toxicity and therefore the expression of genes involved in translation and also in other metabolic basic functions of the cell increased. Dubey *et al*. (2006) transcriptome analysis of the TBT-resistant *P. aeruginosa* 25W evidenced that transcriptional and translational genes are likely affected by TBT toxicity. Meanwhile, Fukushima *et al*. (2009) demonstrated by DNA microarray analysis in the same strain that downregulated genes were related with translational and energy metabolism, evidencing that these mechanisms are firstly affected. Also, TBT exposure leads to the upregulation of ribosomal protein gene, ribosome-modulation factor gene, elongation factor Tu gene and cold-shock protein gene. This indicates that in *P. aeruginosa* 25W*,* TBT affects initially a set of genes and the upregulation of some other genes enhances the translation machinery, maintaining the biosynthesis in the cell (Fukushima *et al*, 2009). Therefore it can be suggested that, *A. molluscorum* Av27 probably have the same resistance mechanism, since the same set of genes/proteins appears to be involved and over-expressed after TBT exposure. Nevertheless, to corroborate these findings DNA transcriptome and microarrays must be carried out for the determination of the down and up regulated genes implicated by TBT exposure in this strain.

From the protocol 2 in one of the bands excised (2nd from the bottom, fig. 8A) above the 14 kDa, by MS analysis a fragment was identified that shows high homology with D-alanine-D-alanine ligase (Ddl). This Ddl is an essential enzyme that catalyses the ligation of D-Ala–D-Ala in the assembly of peptidoglycan precursors, involved in the cell wall organization (Wu *et al*, 2008). The analysis of the SDS-PAGE (fig. 8A) suggests that this enzyme, Ddl, is being overexpressed in response to TBT exposure. This indicates that the preservation and maintenance of the cell wall organization could be one of the TBT-resistance mechanisms. However complementary studies must be carried out to clarify this finding.

Using the protocols above referred it was not possible to identify the SugE-Av27 protein in the present work, and to investigate its involvement in the TBT resistance in *A. molluscorum* Av27. Nevertheless, Jude *et al*. (2004) reported that a multidrug efflux pump cluster gene, TbtABM, is involved in the TBT-resistance in *Pseudomonas stutzeri*. SugE-Av27 protein being a member of the SMR family (Cruz *et al*, 2010) is a proton-dependent multidrug efflux system (Paulsen *et al*, 1996b), thus it has high probability to be involved in TBT resistance in *A. molluscorum* Av27.

Despite the optimization of the two protocols tested for the extraction of membrane proteins in *E. coli*, they were not adequate to the extraction of membrane proteins from *A. molluscorum* Av27. SugE is an integral membrane protein that could be difficult to isolate, since it is surrounded of the cell membrane, it will be necessary to optimize these protocols or to try another approaches for the extraction of this protein. Also, other more efficient detergents can be tested that might help to improve the solubilization of this protein.

2. Heterologous expression of SugE-Av27 protein

To characterize the SugE-Av27 protein higher amounts of the pure protein are needed. The purified protein will help to clarify some aspects such as: What is the conformational stability of the protein under different conditions? How protein-protein interactions and protein-ligand alters the conformation of the protein? How SugE is involved in the resistance of *Aeromonas molluscorum* Av27? Is it involved in other mechanisms of resistance?

To that end, a vector with the gene *sug*E-Av27 was constructed. Two different constructs were made : i) the gene *sug*E was inserted in the vector pET24 with the deletion of the stop codon, so that the protein will be linked to an his-tag; and ii) the stop codon was kept in the *sug*E gene to be used in the Western Blot as a control.

For the heterologous expression of SugE protein, *sug*E gene was inserted on pET24 in *E. coli* BL21 (DE3) with an his-tag. The tag will allow the purification of the heterologous protein by IMAC. The transformation was well succeeded in the two clones, and was confirmed with screening by colony PCR with the appropriate primers (T7prom and T7term), followed by nucleotide sequencing.

The transformation of *E. coli* BL21 (DE3) with the two different plasmids was well succeeded leading to three different clones, BL21 pET24_sugE_HT and BL21 pET24_sugE_STOP.

Proteins from the *E. coli* BL21 pET24_sugE_HT clone induced and uninduced with IPTG were analyzed by SDS-PAGE followed by Western Blot with anti-his-tag. The blot analysis revealed that, at 37°C, the highest expression of SugE protein occurs after 4h of induction. This clone showed poor leaky expression in the cultures grown without IPTG when compared with the induced ones.

The BL21 pET24_sugE_HT was used to the expression of SugE protein with IPTG induction and the western blot analyses showed that there is a high expression of this protein after an incubation period of 4h at 37°C, 180rpm with 1mM of IPTG. Nevertheless, other temperatures and IPTG concentrations must be tested in order to obtain maximum expression level.

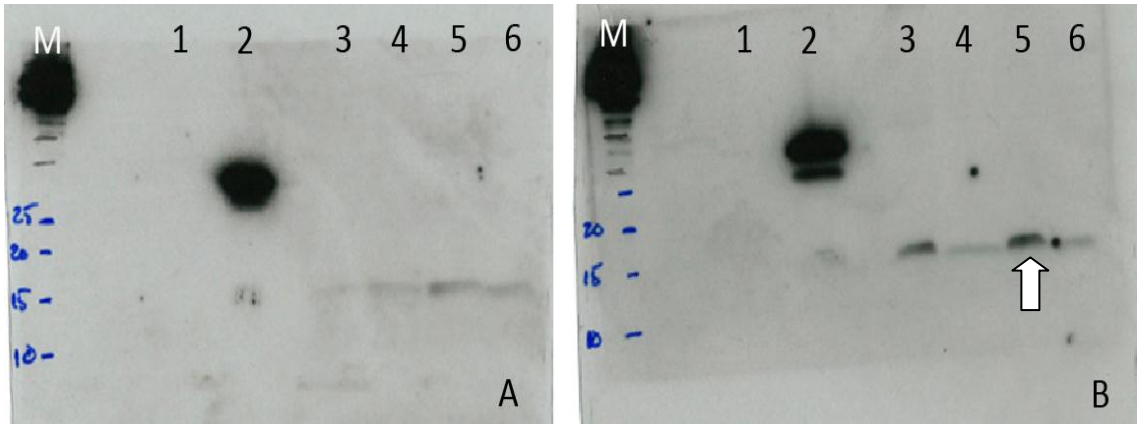


Fig. 9 - Autoradiography of the Western Blot analysis. White arrow indicates the 4h induction detection. M - Bio-Rad Precision Plus Protein dual color standards; A → (1) – negative control, pET24_SugE_STOP (2) – positive control, pETNEK (Wu et al, 2007) (3) – 1 mM IPTG/1h, (4) - without IPTG/1h, (5) – 1 mM IPTG/2h, (6) - without IPTG/2h; (B) → (1) – negative control, pET24_SugE_STOP, (2) – pETNEK (Wu et al, 2007), (3) – 1 mM IPTG/3h, (4) - without IPTG/3h, (5) – 1 mM IPTG/4h, (6)- without IPTG/4h**.**

3. *M. luteus*: a bioindicator species for rapid TBT degradation/toxicity evaluation experiments

With the test of sensitivity it was determined that *M. luteus* is sensible to any of the tested concentrations of TBT (25 µM, 50 µM and 100 µM) and is tolerant to 25 µM of DBT (fig. 10). Thus it can be used as an indicator specie to evaluate the presence of TBT in the culture medium, thus allowing to predict the degradation of TBT to DBT by

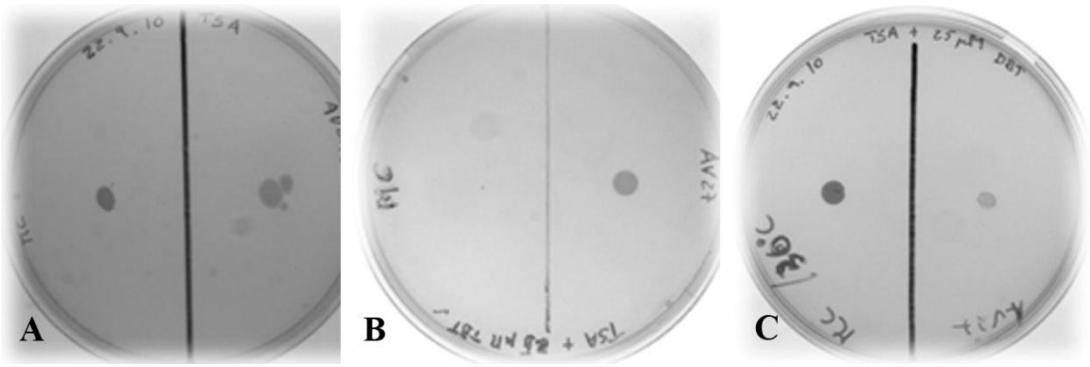


Fig. 10 - Plate assay testing the sensitivity of M. luteus (left in side of plate) and Av27 (right in side of the plate) to TBT and DBT. (A) TSA (B) TSA + 25µM TBT (C) TSA + 25µM DBT

Av27.

In the degradation experiment, using the plate bioassay, it was observed that the inhibition zone in the wells containing the Av27's culture disappear after 54h (fig. 11). So it can be predicted that the toxicity of TBT has been decreased, probably due to the TBT degradation to DBT, since growth of *M. luteus* was observed around the well.

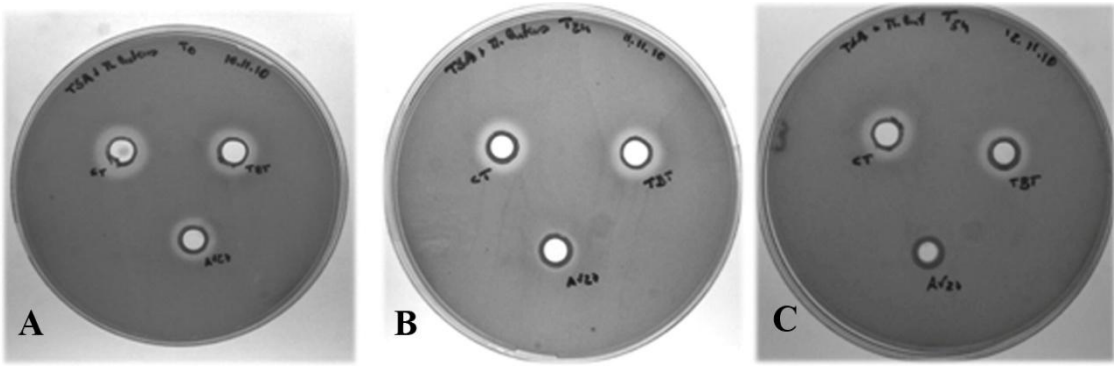


Fig. 11 - Plate assay for the degradation of TBT experiment. (A) Assay made at T_{0h} (B) T_{24h} (C) T_{54h}; CT – 25 µM TBT control; TBT – Av27 incubated with 25 µM TBT; Av27 – Av27 incubated without TBT.

These experiments need to be repeated and some parameters must be changed, for instance i) different culture media like Tryptic Soy Broth, Marine Broth and Minimal Medium, ii) addition of selected different nutrients and iii) variations of pH, salinity, temperature and bacterial cell density. Furthermore, samples withdrawn from the liquid assays will be then sent to organotin content analyses by GC-MS in order to confirm and evaluate the TBT degradation. Those results are fundamental to prove the sensitivity of the method as well as to confirm its applicability as a rapid and feasible method to evaluate TBT degradation.

E. Conclusions

In the present work, one of the aims was to verify the over-expression of SugE protein, involved in the resistance to TBT in *A. molluscorum* Av27. This aim was not completely achieved. It seems that the protocols used for the membrane protein extraction were not effective for this strain or to extract the target protein being studied, since MS results revealed the presence mainly of cytoplasmic proteins in the protein extracts. However, several proteins involved in metabolic pathways essential for cell survival, such as glycolysis, ATP synthesis and protein biosynthesis could be identified. Additionally, a cell wall biosynthesis related protein, Ddl, is suggested to be over-expressed in this strain exposed to TBT.

For the characterization of SugE protein, it is necessary to have this protein in high amounts and in its pure form. For this purpose, *E. coli* cells were transformed with the vector pET24 where the *sug*E gene was inserted. The fusion protein, SugE_his-tag protein, seems to have its highest production when the clone is incubated at 37°C, 160rpm for 4h with 1mM of IPTG. Nevertheless, in future work other concentrations of IPTG and other incubation temperatures need to be tested in order to achieve the maximum expression of the fusion protein.

Since organotin analysis is time consuming and associated with high costs, the use of *M. luteus* as an indicator specie to evaluate TBT degradation/toxicity seems to be a rapid, simple and useful method, prior to GS-MS analysis. After validation more tests, with the variation of physical and chemical parameters, can be performed in order to optimize the TBT degradation by *A. molluscorum* Av27.

This study provides the basis for future work with *A. molluscorum* Av27 and its application in bioremediation of TBT contaminated sites. Thus the following conclusions can be withdrawn:

- Further studies are needed to better clarify the mechanisms behind TBT resistance in *A. molluscorum* Av27, namely those that will evaluate the regulation of genes expressed in response to TBT exposure;

- Heterologous expression in an *E. coli* system, shows to be a suitable approach to express and purify SugE-Av27 protein, for further characterization;

- The use of a bioassay based on *M. luteus* inhibition to evaluate TBT degradation by Av27, shows to be an efficient and rapid method, prior to GC-MS analysis.

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G. Appendix

Appendix 1

Table 1.1 – Recipe of SDS-PAGE gels. [1]stacking gel; [2]running gel

Reagents	Stacking gel 6%	Running gel 16%	Running gel 18%	Running gel 20%
Bis-Acryl (30:0.8)	1 ml	8 ml	6 ml	5.33 ml
0.5M[1]/3M[2] Tris pH 8.0[1], pH 6.8[2]	1.25 ml	5 ml	1.25 ml	1 ml
10% SDS	50 µl	150.5 µl	100 µl	80 µl
MQH_2O	2.616 ml	-	2.58 ml	1.527 ml
15% APS	34 µl	34 µl	50 µl	53 µl
TEMED	5 µl	5 µl	20 µl	8 µl
Urea	-	5.4 g	-	-

Appendix 2

Table 2.1 - PCR primers sequences for the amplification of *sug*E-Av27 gene and for the screening of positive clones. The shadow zone in the sequence represents the enzyme restriction sites.

Primer	Sequence
SugE_NdeI_fw	5'- AAGGAGATATACATATGTTCATGCCCTGGATATTGCTG-3'
SugE_XhoI_his_rv	5'- GGTGGTGGTGCTCGAGACCGATGGCTTTGAGACCCAG-3'
SugE_XhoI_rv	5'- GGTGGTGGTGCTCGAGTCAACCGATGGCTTTGAGACCCAG-3'
T7prom	5' - GCTAGTTATTGCTCAGCG - 3'
T7term	5' - TAATACGCATCACTATAGGG - 3'

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Table 2.2 - PCR program for the amplification of the *sug*E-Av27 gene (A); screening of positive clones (B); and (C) amplification with the T7term and T7prom primers.

		A		B		C	
		Temperature	**Time**	**Temperature**	**Time**	**Temperature**	**Time**
Initial denaturation		95°C	5 min	95°C	5 min	95°C	5 min
Cycle program : 30X	**Denaturation**	95°C	30 s	95°C	30 s	95°C	30 s
	Annealing	54°C	30 s	54°C	30 s	52°C	30 s
	Extension	68°C	45 s	72°C	45 s	72°C	45 s
Final extension		68°C	10 min	72°C	10 min	72°C	10 min

Appendix 3

Table 3.1 – List of proteins indentified in the extracts from Av27 grown in the presence of 500µM TBT. Red square indicates homologous proteins identified in the experiments of Dubey *et al.* (2006) and Fukushima *et al.* (2009)

Protein	Accession Nº	MW (Da)	Biologic process
Protocol 1			
50S ribosomal protein L29	RL29_AERS4	7189,85	Protein biosynthesis
30S ribosomal protein S20	RS20_AERSA	7967,41	Protein biosynthesis
30S ribosomal protein S21	RS21_AERS4	8467,74	Protein biosynthesis
DNA-binding protein HU-alpha	DBHA_AERHY	9393,13	Protein biosynthesis
30S ribosomal protein S19	RS19_AERS4	10433,74	Protein biosynthesis
50S ribosomal protein L24	RL24_AERHH	11302,40	Protein biosynthesis
50S ribosomal protein L21	RL21_AERHH	11392,11	Protein biosynthesis
50S ribosomal protein L22	RL22_AERS4	12386,88	Protein biosynthesis
50S ribosomal protein L19	RL19_AERS4	13156,27	Protein biosynthesis
30S ribosomal protein S13	RS13_AERS4	13192,30	Protein biosynthesis
50S ribosomal protein L14	RL14_AERS4	13394,29	Protein biosynthesis
30S ribosomal protein S11	RS11_AERS4	13826,36	Protein biosynthesis
30S ribosomal protein S9	RS9_AERS4	14759,98	Protein biosynthesis
50S ribosomal protein L15	RL15_AERS4	15039,27	Protein biosynthesis
30S ribosomal protein S6	RS6_AERS4	15067,42	Protein biosynthesis
50S ribosomal protein L13	RL13_AERS4	15798,51	Protein biosynthesis

30S ribosomal protein S5	RS5_AERS4	17328,29	Protein biosynthesis
30S ribosomal protein S7	RS7_AERS4	17408,39	Protein biosynthesis
50S ribosomal protein L6	RL6_AERHH	18511,03	Protein biosynthesis
50S ribosomal protein L5	RL5_AERS4	20227,63	Protein biosynthesis
50S ribosomal protein L4	RL4_AERHH	22182,91	Protein biosynthesis
50S ribosomal protein L3	RL3_AERS4	22349,83	Protein biosynthesis
30S ribosomal protein S4	RS4_AERS4	23378,50	Protein biosynthesis
50S ribosomal protein L1	RL1_AERHH	24585,24	Protein biosynthesis
30S ribosomal protein S3	RS3_AERHH	26117,20	Protein biosynthesis
30S ribosomal protein S2	RS2_AERS4	26894,77	Protein biosynthesis
50S ribosomal protein L2	RL2_AERS4	29974,11	Protein biosynthesis
RNA polymerase subunit alpha	RPOA_AERS4	36173,00	Transcription
Elongation factor Tu	EFTU_AERS4	43272,15	Protein biosynthesis
Na(+)-NQR subunit F	NQRF_VIBVY	44916,13	Sodium ion transport
Enolase	ENO_TOLAT	45525,47	Glycolysis
ATP-binding subunit ClpX	CLPX_AERS4	46434,22	Protein folding
NAD(P) transhydrogenase subunit beta	PNTB_ECOL6	48691,52	
ATP synthase subunit beta	ATPB_ALTMD	49946,65	ATP synthesis coupled proton transport
Sulfate adenylyltransferase subunit 1	CYSN_AERS4	52190,24	Sulfur metabolism
ATP synthase subunit alpha	ATPA_AERS4	55173,97	ATP synthesis coupled proton transport
phosphoglycerate mutase	GPMI_AERS4	55240,39	Glycolysis
60 kDa chaperonin - GroEL protein	CH60_AERHH	57144,64	Protein refolding
GMP synthase	GUAA_AERS4	58475,62	Purine biosynthesis
Chaperone protein dnaK	DNAK_PROMH	69240,56	Stress response

Chaperone protein htpG	HTPG_AERS4	71684,29	Stress response
Threonyl-tRNA synthetase	SYT_AERS4	72869,54	Protein biosynthesis
Polyribonucleotide nucleotidyltransferase	PNP_AERS4	76394,63	mRNA catabolic process
Elongation factor G	EFG_AERS4	77448,38	Protein biosynthesis
Translation initiation factor IF-2	IF2_AERS4	98315,86	Protein biosynthesis
RNA polymerase subunit beta	RPOB_AERS4	150158,14	Transcription
RNA polymerase subunit beta-beta	RPOBC_WOLTR	317901,84	Transcription

Protocol 2			
Glyceraldehyde-3-phosphate dehydrogenase	G3P1_SYNY3	36123,36	Glycolysis
ATP synthase subunit alpha	ATPA_AERS4	55173,97	ATP synthesis
ATP synthase subunit beta	ATPB_PSEA8	55211,82	ATP synthesis
Chaperone protein dnaK	DNAK_AERS 4	69514,81	Chaperones and heat shock proteins
Translation initiation factor IF-2	IF2_AERHH	98109,74	Protein biosynthesis
Pyruvate dehydrogenase E1 component	ODP1_ECOLI	99605,95	Glycolysis
Elongation factor Tu	EFTU_AERS4	43272,15	Protein biosynthesis
Elongation factor G	EFG_AERS4	77448,38	Protein biosynthesis
Elongation factor G	EFG_PHOLL	77649,19	Protein biosynthesis
Elongation factor Ts	EFTS_AERS4	31140,09	Protein biosynthesis
RNA polymerase subunit alpha	RPOA_AERS4	36173,00	Transcription
RNA polymerase subunit beta	RPOC_AERS4	158099,48	Transcription
50S ribosomal protein L34	RL34_AERS4	5046,91	Protein biosynthesis
50S ribosomal protein L33	RL33_SACD2	5981,20	Protein biosynthesis

50S ribosomal protein L32	RL32_AERS4	6212,28	Protein biosynthesis
50S ribosomal protein L30	RL30_AERS4	6606,54	Protein biosynthesis
50S ribosomal protein L29	RL29_AERS4	7189,85	Protein biosynthesis
30S ribosomal protein S20	RS20_AERHY	7967,41	Protein biosynthesis
30S ribosomal protein S21	RS21_AERS4	8467,74	Protein biosynthesis
30S ribosomal protein S17	RS17_AERHH	9393,13	Protein biosynthesis
30S ribosomal protein S15	RS15_AERS4	10076,47	Protein biosynthesis
30S ribosomal protein S19	RS19_AERS4	10433,74	Protein biosynthesis
50S ribosomal protein L24	RL24_AERS4	11292,31	Protein biosynthesis
30S ribosomal protein S14	RS14_AERS4	11522,26	Protein biosynthesis
50S ribosomal protein L22	RL22_AERS4	12386,88	Protein biosynthesis
50S ribosomal protein L18	RL18_AERS4	12471,74	Protein biosynthesis
50S ribosomal protein L19	RL19_AERS4	13156,27	Protein biosynthesis
50S ribosomal protein L14	RL14_AERS4	13394,29	Protein biosynthesis
50S ribosomal protein L20	RL20_AERS4	13417,60	Protein biosynthesis
30S ribosomal protein S11	RS11_AERS4	13826,36	Protein biosynthesis
30S ribosomal protein S8	RS8_AERS4	13992,52	Protein biosynthesis
50S ribosomal protein L17	RL17_AERS4	14307,66	Protein biosynthesis
30S ribosomal protein S9	RS9_AERS4	14759,98	Protein biosynthesis
50S ribosomal protein L11	RL11_AERS4	14979,92	Protein biosynthesis
50S ribosomal protein L15	RL15_AERS4	15039,27	Protein biosynthesis
50S ribosomal protein L9	RL9_AERS4	15319,33	Protein biosynthesis
50S ribosomal protein L16	RL16_AERHH	15372,32	Protein biosynthesis
50S ribosomal protein L13	RL13_AERS4	15798,51	Protein biosynthesis

30S ribosomal protein S5	RS5_AERS4	17328,29	Protein biosynthesis
30S ribosomal protein S7	RS7_AERS4	17408,39	Protein biosynthesis
50S ribosomal protein L10	RL10_AERS4	17699,43	Protein biosynthesis
50S ribosomal protein L6	RL6_AERHH	18511,03	Protein biosynthesis
50S ribosomal protein L5	RL5_AERS4	20227,63	Protein biosynthesis
50S ribosomal protein L3	RL3_AERHH	22408,93	Protein biosynthesis
30S ribosomal protein S4	RS4_AERS4	23378,50	Protein biosynthesis
50S ribosomal protein L1	RL1_AERS4	24588,21	Protein biosynthesis
30S ribosomal protein S3	RS3_AERHH	26117,20	Protein biosynthesis
30S ribosomal protein S2	RS2_AERS4	26894,77	Protein biosynthesis
50S ribosomal protein L2	RL2_AERS4	29974,11	Protein biosynthesis

Table 3.2 - List of proteins identified in the bands excised from SDS-PAGE analysis. Red square indicates homologous proteins identified in the experiments of Dubey *et al*. (2006) and/or Fukushima *et al*. (2009). Bands were numbered from bottom to top.

Band Nº	Protein	Accession Nº	MW (Da)	Pathway
Protocol 1				
1	**50S ribosomal protein L29**	RL29_AERS4	7189,9	Protein Biosynthesis
3	**50S ribosomal protein L28**	RL28_AERHH	8848,8	Protein Biosynthesis
4	**50S ribosomal protein L27**	RL27_AERS4	9061,9	Protein Biosynthesis
5	**50S ribosomal protein L19**	RL19_AERS4	13156,3	Protein Biosynthesis
6	**30S ribosomal protein S11**	RS11_AERS4	13826,4	Protein Biosynthesis
7	**50S ribosomal protein L16**	RL16_AERS4	15386,3	Protein Biosynthesis

8	**50S ribosomal protein L13**	RL13_AERS4	15798,5	Protein Biosynthesis
8	**30S ribosomal protein S5**	RS5_AERS4	17328,3	Protein Biosynthesis
9	**50S ribosomal protein L4**	RL4_AERS4	22196,9	Protein Biosynthesis
10	**50S ribosomal protein L3**	RL3_AERS4	22349,8	Protein Biosynthesis
10	**30S ribosomal protein S4**	RS4_AERS4	23378,5	Protein Biosynthesis
11	**50S ribosomal protein L2**	RL2_AERS4	29974,1	Protein Biosynthesis
12	**DNA-directed RNA polymerase subunit alpha**	RPOA_AERS4	36173,0	Transcription

Protocol 2				
2	**D-alanine-D-alanine ligase**	DDL_AERHH	36320,5	Cellular cell wall organization
3	**Peptide chain release factor 1**	RF1_ZYMMO	39296,2	Protein Biosynthesis
5	**30S ribosomal protein S8**	RS8_AERS4	13992,5	Protein biosynthesis
5	**30S ribosomal protein S14**	RS14_AERS4	11522,3	Protein biosynthesis
6	**Elongation factor Tu**	EFTU_AERS4	43272,1	Protein Biosynthesis
7	**RNA polymerase subunit alpha**	RPOA_AERS4	36173	Transcription
7	**Phosphoglycerate kinase**	PGK_AERS4	40439,1	Glycolysis