Universidade de Aveiro Departamento de Química
Ano 2013

Raquel Sofia Mesquita Rodrigues

Análise do transcriptoma de *A. molluscorum* Av27 após exposição ao TBT

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, Ramo de Biotecnologia Molecular, realizada sob a orientação científica da Doutora Sónia Mendo, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro, e da Doutora Andreia Cruz, Estagiária em Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Tributilestanho (TBT), *Aeromonas molluscorum* Av27, resistência, degradação, transcriptoma, RNA-Sequencing, PCR em tempo real, expressão diferencial.

resumo

O tributilestanho (TBT) é um composto tóxico com efeitos nefastos para o ambiente. Este composto foi utilizado durante vários anos como componente de tintas anti-vegetativas aplicadas nos cascos dos barcos sendo, por isso, reconhecido mundialmente como uma das fontes de contaminação de ambientes aquáticos. Atualmente, o uso destas tintas está proibido em alguns países, verificando-se uma diminuição na concentração de TBT no ambiente. Apesar disso, devido à estabilidade e persistência deste composto (principalmente nos sedimentos), a poluição por TBT continua a ser preocupante.

Aeromonas molluscorum Av27 foi isolada no sedimento da Ria de Aveiro, num local contaminado por TBT. Esta bactéria é tolerante a concentrações elevadas de TBT (até 3 mM) e é capaz de degradar o composto nos seus derivados menos tóxicos, DBT e MBT. Com o intuito de conhecer o(s) mecanismo(s) molecular(es) que estão na base destas propriedades, procedeu-se à análise do transcriptoma desta estirpe por pirosequenciação. Para isso, para além da condição controlo (sem TBT), as células foram expostas a 5 e 50 μM de TBT até atingirem o meio da fase exponencial. A validação dos resultados de pirosequenciação foi feita por PCR em tempo real.

De uma forma geral, a análise dos transcriptomas de *A. molluscorum* Av27 revelou a presença de diversos genes sobre-expressos após exposição ao TBT. Os genes que se relacionam com a atividade enzimática e o transporte/ligação de compostos foram aqueles que sofreram maiores alterações a nível de expressão, propondo-se desta forma o seu envolvimento nos mecanismos de resistência e degradação de TBT. Alguns dos genes sobre-expressos identificados codificam para bombas de efluxo e outras proteínas envolvidas na resistência a antibióticos e metais pesados, corroborando a relação entre a resistência a estes compostos e a resistência ao TBT. Para além disso, foi sugerido que proteínas envolvidas na resposta ao stress térmico podem também desempenhar um papel importante na resistência ao TBT. Tendo em conta a análise feita, não foi possível encontrar uma proteína responsável pela degradação do TBT. No entanto, foram detetadas várias proteínas sobre-expressas de função desconhecida. A anotação destas proteínas é de grande importância, uma vez que poderá contribuir para a elucidação do mecanismo de degradação de TBT nesta bactéria.

Em estudos anteriores, demonstrou-se que o gene *sug*E está envolvido no mecanismo de resistência ao TBT em Av27 e que o seu nível de expressão está dependente da fase de crescimento das células. No presente estudo, foi possível confirmar que o gene *sug*E é sub-expresso na presença de TBT quando as células atingem a sua fase exponencial.

Foi ainda possível notar que genes relacionados com a transcrição estão sub-expressos após exposição ao TBT, indicando que este composto afeta a transcrição genética.

O estudo detalhado dos genes identificados neste trabalho, potencialmente envolvidos no mecanismo de resistência e/ou degradação do TBT, poderá contribuir para a compreensão destes mecanismos em *A. molluscorum* Av27, assim como noutros organismos procariotas.

keywords

Tributyltin (TBT), *Aeromonas molluscorum* Av27, resistance, degradation, transcriptome, RNA-Sequencing, Real time PCR, differential expression.

abstract

Tributyltin (TBT) is a toxic compound with a negative impact to the environment. This compound was used for several years as a component of antifouling paints applied to ship hulls, thus contaminating several aquatic environments worldwide. Currently, the use of these paints is prohibited in several countries, and there has been a consequent decrease of TBT concentration in the environment. However, due to the stability and persistence of the compound (mainly in the sediments), TBT pollution remains a serious problem.

Aeromonas molluscorum Av27 was isolated in the sediments of Ria de Aveiro, in a TBT contaminated site. This bacterium is tolerant to high TBT concentrations (up to 3 mM) and is able to degrade it into the less toxic compounds DBT and MBT. In order to better understand the molecular mechanism(s) conferring these properties, a transcriptome analysis was carried out. In addition to the control (without TBT), the cells were grown to the mid-log phase in presence of different TBT concentrations (5 and 50 μM). Pyrosequencing analysis was performed in each of the samples. Validation of the transcriptome results was performed by quantitative real-time PCR.

The analysis of the transcriptomes of *A. molluscorum* Av27 revealed that several genes were up-regulated following exposure to TBT. Genes responsible for enzymatic activities and transport/binding were the most affected by TBT exposure and thus, those genes seem to be involved in TBT resistance and degradation. Some efflux pumps and other proteins involved in resistance to antibiotics or heavy metals were found over-expressed when Av27 cells were exposed to TBT, supporting the relationship between the resistance to these compounds and resistance to TBT. Furthermore, a possible role of heat-shock proteins in TBT resistance in *A. molluscorum* Av27 was also suggested. So far, the analysis of the transcriptome didn't allow the identification of the protein responsible for TBT degradation in *A. molluscorum* Av27. However, several proteins of unknown function were over-expressed in the presence of the toxic compound. The annotation of such proteins is important, since it might help to elucidate the TBT degradation mechanisms in this bacterium.

Previous studies demonstrated that the s*ug*E gene is involved in TBT resistance in Av27 strain, and that its' expression levels depend on the growth phase. Likewise, in the present study, the *sug*E gene was over-expressed when the cells were grown to the mid-log phase.

It was also verified that several transcription-related genes were under-expressed in *A. molluscorum* Av27 following exposure to TBT, suggesting that this compound negatively affects genetic transcription.
Further investigation of the genes potentially involved in TBT resistance/degradation may contribute to a better understanding of these mechanisms in *A. molluscorum* Av27, as well as in other prokaryotes.

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INTRODUCTION

1. Organotin compounds

Organotin compounds (OTs) are organometallic chemicals (with one or more carbon-tin bounds) used in several industrial processes as fungicides, acaricides, disinfectants, ingredients of marine antifouling paints, among others (Cooney and Wuertz 1989; Murata, Takahashi et al. 2008). Besides, OTs can be used as catalysts in the preparation of silicone rubbers and polyurethane foams and also as components in anti-leukemia drugs (Sherman and Huber 1988; Cooney and Wuertz 1989). They are often discharged into the environment causing several problems, such as, for instance, immunotoxic, hepatotoxic and neurotoxic effects in fish and mammals (Hoch 2001; Murata, Takahashi et al. 2008).

Organic derivatives of tin are represented by the general formula R_3SnX_{4-n} and are characterized by the presence of covalent bonds between three carbon atoms and a tin atom (Sn^{4+}). In general, in the previous formula, *X* is an anion and it influences the physicochemical properties of the compound; *R* represents an alkyl or aryl group and *n* a number from 1 to 4. Tin compounds are toxic, and its toxicity depends on the nature of the alkyl radical (higher for alkyls than aryl groups). Trisubstituted organotins are usually more toxic than the di-, mono- or tetrasubstituted (toxicity in decreasing order: $R_3SnX > R_2SnX_2 > RSnX_3 > R_4Sn$) (Cooney and Wuertz 1989; Pain and Cooney 1998; Alzieu 2000).

Inorganic forms of tin can be toxic to microorganisms, but they are apparently harmless to humans. Organotins, on the other hand, are more lipophilic, penetrating the biological membranes more easily and accumulating in lipid-rich tissues or organelles (Cooney and Wuertz 1989; Dubey and Roy 2003). These compounds can be toxic to aquatic organisms even at concentrations as low as 1-2 ng/L (White, Tobin et al. 1999; Hoch 2001). OTs affect many eukaryotic and prokaryotic organisms, including higher-trophic level aquatic organisms (e.g. marine mammals) and humans (immune and endocrine systems), where they are usually found in the blood and the liver (Cooney and Wuertz 1989; Alzieu 2000; Antizar-Ladislao 2008). Human exposure to organotins may occur due to the use of these compounds in PVC food wrappings, bottles and rigid potable water pipes (Dubey and Roy 2003).

2. Tributyltin

Tributyltin (TBT) (Figure 1) is a hydrophobic and positively charged organic derivative of tin. It is represented by the formula $C_{12}H_{27}Sn^{+}$ and its molecular weight is 290.06 (Jude, Arpin et al. 2004; Antizar-Ladislao 2008).

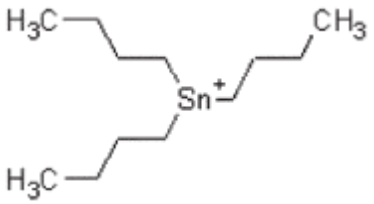


Figure 1 - TBT chemical structure. Adapted from Antizar-Ladislao (2008).

TBT is trisubstituted, which is the most toxic form of tin derivative compounds (Cooney and Wuertz 1989). There are several forms of TBT, such as tributyltin oxide (TBTO), tributyltin chloride (TBTCl) and tributyltin fluoride (TBTF), among others. These compounds are a subgroup of the trialkyl organotin family (Dubey and Roy 2003).

Tributyltin presents broad-spectrum biocidal properties, so it can be used as fungicide, bactericide, pesticide, wood preservative, PVC stabilizer and as a component of antifouling paints (Ranke and Jastorff 2000; Cruz, Caetano et al. 2007; Kingtong, Chitramvong et al. 2007). It can also be used as slime control in paper mills, in the disinfection of circulating industrial cooling waters and to prevent the attachment of barnacles and slime on fishing nets (Antizar-Ladislao 2008; Murata, Takahashi et al. 2008). The use of TBT in antifouling paints is the most economically relevant, since the growth of organisms in boat and ship hulls increases the friction of boat in the water, leading to a reduction of the speed and consequent increase in the fuel consumption (Cooney and Wuertz 1989; Karlsson and Eklund 2004). After being introduced in the 1960s, and during the 1970s and early 1980s, TBT was the antifouling agent of choice due to its effectiveness and longevity (Bennett 1996; Pain and Cooney 1998; Dubey and Roy 2003). In these paints, TBT is usually chemically bonded in a copolymer resin system. The bound is based on an organotin-ester linkage. The release of TBT from the paints is slow and controlled, and it occurs because the organotin-ester link becomes hydrolysed when in contact with sea waters (Evans 1999).

Introduction

Due to its applications, TBT is a common contaminant in aquatic ecosystems. It rapidly adsorbs to suspended particles (due to its preferential partitioning onto colloidal and particulate surfaces), which eventually deposit and consolidate (Batley 1996). Besides, it is usually absorbed by bacteria and algae, entering the food chain, and it eventually accumulates in higher organisms (fish, water birds and mammals) (Gadd 2000; Borghi and Porte 2002; Luan, Jin et al. 2006; Antizar-Ladislao 2008). In humans, TBT is probably ingested, since it contaminates organisms that can be included in human diet and it is not destroyed by common cooking practices (Short and Thrower 1986; Antizar-Ladislao 2008).

TBT toxicity is related to its partition coefficient in octanol/water. This coefficient quantifies the affinity to lipids - higher coefficient means greater hydrophobicity and toxicity. TBT is hydrophobic, affecting negatively the integrity of biological membranes (Mackay 1982; Cooney and Wuertz 1989; Dubey and Roy 2003; Jude, Arpin et al. 2004). Thus, the physiological functions of the lipid membranes may be compromised in both eukaryotes and prokaryotes (Cooney and Wuertz 1989). Moreover, this compound interferes with the energy transduction apparatus and acts as an ionophore, facilitating halide-hydroxyl exchanges (Selwyn 1976; Cooney and Wuertz 1989; Dubey and Roy 2003). Malformations of the mitochondrial membranes are also observed, since TBT inhibits the oxidative phosphorylation (Alzieu 2000). Tributyltin also causes inhibition of photophosphorylation (affecting chloroplasts), inhibition of ion pumps including the Na^+/K^+ ATPase, inhibition of the cytochrome P450 system, disturbance of Ca^{2+} homeostasis and induction of apoptosis in thymocytes, affecting the immune system (Fent 1996; Ranke and Jastorff 2000).

TBT is toxic even at low concentrations. In fact, approximately 10% of the species of several groups are affected by TBT in concentrations that range from 5 ng/L (zooplankton) to 2 pg/L (fish) (Halla, Scott et al. 1998). Effects on molluscs are observed when TBT is present at concentrations < 1 ng/L (Alzieu 2000).

As a result of the negative impacts of TBT, the use of TBT-based paints on small boats (<25 m) was banned in France (1982) (Cooney and Wuertz 1989), United Kingdom (1987) (Barroso, Moreira et al. 2000) and USA (1988) (Murata, Takahashi et al. 2008). TBT restrictions are mainly focused on small boats because these vessels spend most of the time near shore or in the harbor, increasing the risk of tributyltin accumulation in the sediments. On the other hand, ships larger than 25 m are more frequently at deep sea (Cooney and Wuertz 1989). Later, in the 90s, TBT production, use and exportation were prohibited in

other developed countries. In Portugal, the Portuguese Navy banned the use of TBT from their ships in 1992 and in 1993 the use of TBT-containing paints on small boats was also forbidden (Barroso, Moreira et al. 2000). The application of TBT-containing paints on boats from the European Union was prohibited in 2003, and in 2008 the use of TBT was banned by the International Maritime Organization (Antizar-Ladislao 2008; Mimura, Sato et al. 2008).

Before the restrictions introduced in 1982, TBT levels in seawater generally ranged between 50 and 500 ng/L in North American and European marinas. In 2000, the maximum concentration recorded in marina waters rarely exceeded 200 ng/L (average: 42 ng/L) along the English Channel and Atlantic coasts. In harbor sediments, TBT concentrations ranged between 1 and 2 mg/Kg dry weight (Alzieu 2000). These values show that despite the widespread ban of the use of TBT on smaller craft, this compound still causes several environmental problems. This happens due to tributyltin's slow degradation and to its continuous use on large vessels (Batley 1996; Matthiessen and Gibbs 1998). TBT has low solubility in seawater and its half-life in this environment is highly variable, depending on pH, temperature, turbidity and light (Alzieu 2000). In general, the half-life of TBT in seawater is considered to be of a few days to a few weeks and in sediments of 4-6 months. In the sediment core, the half-life is estimated to go up to several years (Stewart and Mora 1990; Seligman, Maguire et al. 1996; Ranke and Jastorff 2000). In estuarine systems, TBT usually persists for 6-7 days (28 ºC) in the water and 1.9 to 3.8 years in deep, anoxic sediments (Batley 1996; Cruz 2012). Accordingly, the sediment is a reservoir of the organotin compound, which persists stable for long periods of time (Stewart and Mora 1990; Langston and Pope 1995). Organotin's natural degradation may occur due to UV irradiation, chemical, biological or thermal cleavage of the Sn-C bond, but the predominant mechanism is biodegradation by microorganisms (Blunden, Hobbs et al. 1984; Watanabe, Sakai et al. 1992). Besides, degradation is faster if the phytoplankton population is high (Lee 1985; Cooney and Wuertz 1989; Watanabe, Sakai et al. 1992). Dibutyltin (DBT) and monobutyltin (MBT) are the degradation products of TBT. These degradation products are less toxic than TBT (Pain and Cooney 1998; Cruz, Caetano et al. 2007).

2.1 Negative effects of TBT

Exposure to TBT has negative effects in both prokaryotes and eukaryotes (Cooney and Wuertz 1989). In *Escherichia coli*, for instance, TBT affects several reactions involved in growth, solute transport, biosynthesis of macromolecules and activity of transhydrogenases (Singh and Singh 1985; Singh 1987). TBT can also interact with cytosolic enzymes (White, Tobin et al. 1999) and inhibit cell growth and amino acids uptake (Singh and Bragg 1979; Jude, Capdepuy et al. 1996). In fungi, morphological changes and increased melanin synthesis were observed in colonies of *Penicillium funiculosum*, *Phoma glomerata* and *Aureobasidium pullulans* (Newby and Gadd 1988).

Cooney and Wuertz (1989) reported that some algae species suffered from growth inhibition or even death when exposed to tributyltin. The growth of *Skeletonema costatum* and *Thalassiosira pseudonana*, for instance, was inhibited when exposed to TBT (Walsh, McLaughlan et al. 1985).

TBT interacts with mitochondria and chloroplasts in both fungi and microalgae, affecting the organelles' correct function (Cooney and Wuertz 1989).

In abalone (Horiguchi, Kojima et al. 2002), ivory shells (Horiguchi, Kojima et al. 2005) and gastropods (Matthiessen and Gibbs 1998; Horiguchi 2006), exposure to TBT induces imposex, the superimposition of male characters onto female organisms, with possible appearance of penis and vas deferens (Smith 1971). This phenomenon is a consequence of endocrine disruption, since TBT probably acts as a competitive inhibitor of cytochrome P450-mediated aromatase or inhibits testosterone metabolism and excretion, leading to an increase of the hormone's concentration levels and consequent development of male sex organs (Matthiessen and Gibbs 1998; Mimura, Sato et al. 2008). The degree of masculinization depends on the concentration of TBT; higher concentrations may lead to breeding inhibition, causing a population decline and eventual extinction. These changes are irreversible and the end-result of these masculinization processes varies according to the species (Alzieu 2000). A similar syndrome named intersex was observed in *Littorina littorea*, consisting on alterations in the pallial oviduct, followed by the appearance of male characteristics in the oviduct (Bauer, Fioroni et al. 1995). Imposex and intersex have also been observed in Ria de Aveiro (Portugal), affecting several species, namely *Nucella*

lapillus, *Nassarius reticulatus*, *Hydrobia ulvae* and *Littorina littorea* (Barroso, Moreira et al. 2000).

Some oyster species are affected even by low TBT concentrations (2 ng/L), presenting abnormalities in shell formation and a deficient larval development (Edouard and Rene 1983-1985; Alzieu 2000). Bivalve reproduction is also altered by the presence of TBT (Thain and Waldock 1986).

It is recognized that TBT affects several organisms. However, molluscs are known to be the most sensitive species to TBT exposure, since the low activities of cytochrome P450 and mixed function oxidases observed in these organisms lead to slow TBT metabolism rate and consequent bioaccumulation (Lee 1991; Alzieu 2000).

In mammals, TBT affects different organs and causes diseases in the nervous, endocrine and immune systems. In humans, the toxic compound seems to cause irritations in eyes and skin, potentially leading to severe dermatitis (Alzieu 2000; Antizar-Ladislao 2008; Akiyama, Iwaki et al. 2011).

2.2 TBT resistance and degradation in bacteria

As above mentioned, TBT is toxic to many organisms. However, some eukaryotic and prokaryotic species developed resistance mechanisms. Some hypotheses have been proposed to explain these mechanisms in bacteria (Dubey and Roy 2003):

- degradation into DBT and MBT (less toxic organotin compounds) by dealkylation mechanisms (Clark, Sterritt et al. 1988; Pain and Cooney 1998);
- transport of the compound to the exterior of the cell through efflux pumps or other membrane proteins (Jude, Arpin et al. 2004);
- metabolic utilization of TBT as carbon source (Kawai, Kurokawa et al. 1998; Cruz, Caetano et al. 2007);
- bioaccumulation of the biocide without cell breakdown, using metallothionein-like proteins (Blair, Olson et al. 1982; Fukagawa, Konno et al. 1994).

Both Gram negative and Gram positive bacteria can be resistant to TBT. However, probably due to the different architecture of cell walls, Gram negative bacteria seem to be more resistant than the former (Cruz, Caetano et al. 2007). Since some resistant Gram positive bacteria have been detected, the resistance mechanism is possibly related not only

to the external membrane, but also to the cytoplasmic membrane and/or to the intracellular environment (White, Tobin et al. 1999; Mendo, Nogueira et al. 2003; Cruz, Caetano et al. 2007).

Miller, Wuertz et al (1995) studied the role of plasmids in the resistance of a bacterium to TBT. The authors isolated several strains highly resistant to TBT that didn't have any plasmids, indicating that TBT resistance is not necessarily plasmid-encoded. However, the successful introduction of the plasmid pUM505 from *Pseudomonas aeruginosa* PAO1 (a TBT-resistant strain) into *Beijerinckia* sp. MC-27 (a TBT-sensitive strain) led to an increased TBT tolerance. Thus, these results suggest that plasmids may play some role in TBT resistance in some species.

In *Alteromonas* sp. M-1, the gene responsible for the TBT-resistance was considered to be chromosomal, since no plasmids were detected in this strain. During the same study, an ORF was identified and its product was assumed to be part of a cluster of membrane proteins involved in transport (transglycosylases) (Fukagawa and Suzuki 1993). Despite that, it was also concluded that more than one gene is involved in the resistance mechanism exhibited by this strain (Fukagawa, Konno et al. 1994).

In *Pseudomonas stutzeri* 5MP1, TBT resistance was found to be associated with an operon, *tbtABM*. The proteins encoded by this operon show homology with efflux pump proteins. Besides TBT, these efflux pumps can also export antibiotics, such as nalidixic acid, chloramphenicol and sulfamethoxazole (Jude, Arpin et al. 2004).

Another efflux pump, AheABC, is involved in TBT resistance in *Aeromonas hydrophila* ATCC7966 (Hernould, Gagné et al. 2008).

Fukushima and co-workers (2009) used *Pseudomonas aeruginosa* 25W, which is highly resistant to TBT, to perform gene expression studies. Some genes were up-regulated upon exposure to the toxic compound, as for instance genes that encode for two ribosomal proteins, a conserved hypothetical protein and cytochrome c550. Several genes were found to be down-regulated and these encode for a ribosomal protein, a ribosome-modulation factor, a cold-shock protein and the elongation factor Tu. It was shown that high TBT concentrations are toxic, leading to the inhibition of the transcription of those genes. The authors suggest that the resistance mechanism is related to the protection and increase of protein synthesis. Furthermore, other gene clusters containing membrane transport protein genes are probably involved in TBT resistance as well (Fukushima, Dubey et al. 2009).

In Ria de Aveiro (Portugal), a TBT-resistant estuarine bacterium, *Aeromonas molluscorum* Av27, was isolated. This bacterium degrades TBT and uses it as carbon source (Cruz, Caetano et al. 2007). The same authors identified a gene, *sug*E, that was shown to be involved in TBT resistance. This gene was over-expressed upon exposure to 500 μM of TBT, when cells were grown to the early logarithmic growth phase. On the other hand, in later growth phases (logarithmic and stationary), the same gene was shown to be under-expressed upon exposure to the toxic compound, suggesting that SugE is involved in a quick response to TBT (Cruz, Micaelo et al. 2013). SugE is included in the small multidrug resistance (SMR) family, that is shown to transport lipophilic drugs, such as TBT (Sikora and Turner 2005).

Several studies showed no correlation between TBT resistance patterns in organisms isolated from more and less polluted areas. Thus, there are probably other factors besides the presence of TBT that select for a TBT-resistant population (Wuertz, Miller et al. 1991; Jude, Capdepuy et al. 1996; Cruz, Caetano et al. 2007).

Metal and antibiotic resistance are often associated to TBT resistance. For instance, some isolated TBT-resistant bacteria showed to be resistant to mercury (Fukagawa, Konno et al. 1994) and cadmium (Suzuki, Fukagawa et al. 1992). Besides, some efflux pumps related to TBT resistance are also able to extrude antibiotics (Jude, Arpin et al. 2004; Hernould, Gagné et al. 2008).

Most of the published studies show that resistance mechanisms are efflux-related. However, many marine bacteria and some algae species are capable of degrading organotin compounds, as for instance, TBT (Cooney and Wuertz 1989; Dubey and Roy 2003). For example, *Pseudomonas aeruginosa*, *Alcaligenes faecalis* (Gram negative bacteria), *Tramatis versicolor* and *Chaetomium globosum* (fungi) are able to degrade tributyltin oxide (TBTO) via a dealkylation process (Barug 1981). Furthermore, *Aeromonas molluscorum* Av27 is also able to degrade TBT into less toxic compounds (DBT and MBT) (Cruz, Caetano et al. 2007). Nevertheless, little is known about the mechanisms behind the biodegradation process.

3. Ria de Aveiro, a TBT contaminated estuarine system

Ria de Aveiro is a shallow estuarine system (area at low tide: 43 Km^2; area at high tide: 47 Km^2) located on the north-west coast of Portugal (40º38'N, 8º41'W), with muddy bottom sediments. Ria de Aveiro exchanges water with the sea through the mouth, from which radiate three main channels (S. Jacinto-Ovar, Mira and Ílhavo). The River Vouga accounts for 2/3 of the total mean river input, being the most important river discharging into the Ria de Aveiro (Moreira, Queiroga et al. 1993; Silva 1994; Barroso, Moreira et al. 2000).

The ports, dockyards (located along the main navigation channel) and marinas are important sources of organotin pollution (Figure 2). The naval construction shipyard (ships of up to 2000 t) located on the western bank of the initial part of the S. Jacinto-Ovar channel is also a TBT source (Barroso, Moreira et al. 2000).

In Ria de Aveiro, TBT levels range from <0.6 to 38.5 ng Sn/L in the water (Galante-Oliveira, Oliveira et al. 2009) and about 66 ng Sn/g dry weight in sediments (Laranjeiro, Sousa et al. 2010). Near the ports, dockyards and marinas, TBT levels are higher (water: 28 to 42 ng Sn/L; sediments: 65 to 88 ng Sn/g dry weight) (Barroso, Moreira et al. 2000). Hence, this estuarine system can be considered a moderately TBT-contaminated site. These values are concerning, since the normal functioning of estuarine ecosystems can be severely affected by TBT pollution. As referred above, the negative impacts of the toxic compound can be detected even in low to moderate contaminated sites (Mendo, Nogueira et al. 2003).

As mentioned before, the use of TBT-containing paints was banned in 2008 (Antizar-Ladislao 2008). However, since TBT is stable, it persists in the environment for long periods of time. Hence, TBT pollution in Ria de Aveiro is still a problem of concern (Laranjeiro, Sousa et al. 2010).

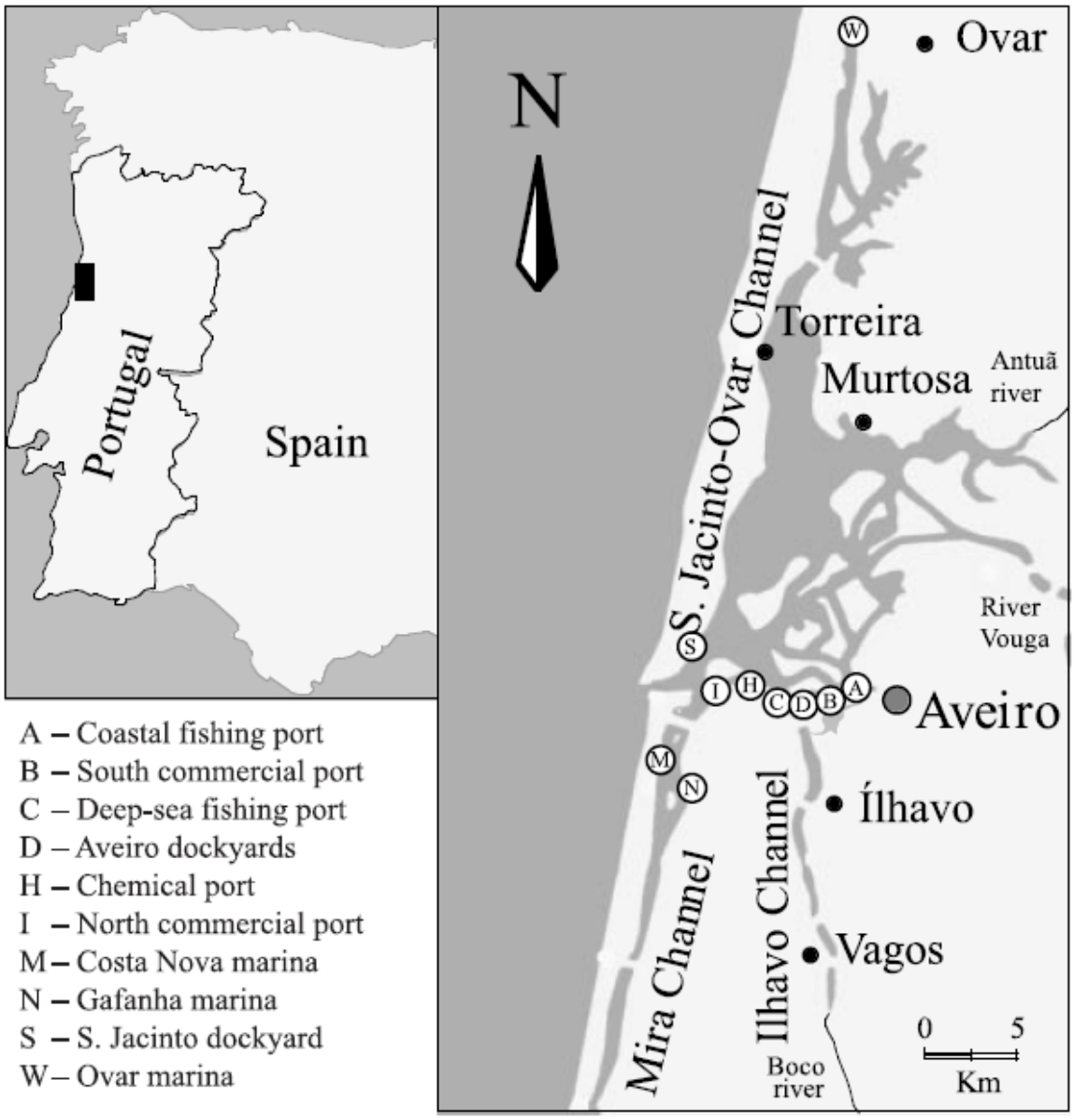


Figure 2 - Location of Ria de Aveiro and main TBT contamination sources (Barroso, Moreira et al. 2000).

4. *Aeromonas molluscorum* Av27

The genus *Aeromonas* (Aeromonadaceae family, *γ-Proteobacteria*) is constituted by Gram negative, rod-shaped, oxidase- and catalase-positive, non-spore forming, glucose-fermenting and facultative anaerobic bacteria. Most species belonging to this genus are motile through the presence of polar flagella and are autochthonous in aquatic environments (Holt, Bergey et al. 1984; Popoff 1984). These bacteria can grow in common culture media, such as TSA (tryptic soy agar), at an optimal temperature of 30ºC (Holt, Bergey et al. 1984).

Aeromonas molluscorum Av27 is an estuarine bacterium isolated from Ria de Aveiro (Portugal). This bacterium is resistant to high concentrations of TBT (up to 3 mM), it is able to degrade it into DBT and MBT and it is also capable of using it as a carbon source (Cruz, Caetano et al. 2007).

It was shown that DBT and MBT (products of TBT degradation) are extruded from the cell and released to the culture media. Possibly, this mechanism involves the capture of TBT into siderophore-like structures, degradation and gradual extrusion from the cell as DBT (Cruz, Caetano et al. 2007).

Aeromonas molluscorum Av27 is resistant to the antibiotics penicillin (10 μg/mL), amoxicillin/clavulanic acid (30 μg/mL) and cephalothin (30 μg/mL). It is also resistant to the vibriostatic agent O/129 (Cruz, Areias et al. 2013).

As far as mobile elements are concerned, five plasmids of 4 Kb, 7 Kb, 10 Kb, 100 Kb and >100 Kb were detected. No class I or class II integrons were detected (Cruz, Areias et al. 2013).

Aeromonas molluscorum Av27 is considered to be safe, since *in vitro* cytotoxic studies revealed no apparent cythopathic effects against mammalian cells (Vero cells) (Cruz, Areias et al. 2013).

As previously mentioned, *Aeromonas molluscorum* Av27 is resistant to TBT and has the ability to degrade it into less toxic compounds. These characteristics increase the interest on this strain for bioremediation, a process utilizing the metabolic potential of microorganisms that is employed to decontaminate polluted environments (Watanabe 2001). Bioremediation processes present several advantages when compared to conventional remediation techniques, such as the reduced costs, it is a non-invasive technique and efficient even when low concentrations of the pollutant is present. Most importantly, it is a permanent solution. However, the application of bioremediation is still limited by the long time required in this process and the fact that it is less predictable than the conventional methods (Perelo 2010).

The identification of genes involved in TBT resistance allowed the construction of a bioreporter to detect TBT in the environment (Cruz 2012). This monitoring method is more effective, quicker, easier and cheaper than the traditional analytical analysis (Durand, Thouand et al. 2003), and also allow to evaluate the bioavailability of the compounds and to assess its biological effects (Hynninen and Virta 2010). In the case of TBT, biomonitoring

also allows to overcome some difficulties encountered when using the chemical measurement, such as the variations observed in the concentration levels at fixed locations over time and the detection of concentrations bellow the detection limit (Barroso, Moreira et al. 2000).

5. Transcriptome analysis

The transcriptome is the total set of transcripts (RNAs) in a cell at a given time. It can be evaluated qualitatively and quantitatively. The amount of transcript is not constant and depends on the environmental conditions (Wang, Gerstein et al. 2009). The study of the transcriptome can provide information about the functional elements of the genome (promoters, transcription start sites, open reading frames, regulatory noncoding regions, untranslated regions and transcription units). Besides, some regulatory mechanisms can be elucidated (Sorek and Cossart 2010; Vliet 2010; Febrer, McLay et al. 2011).

In order to better understand the TBT resistance and degradation mechanisms, it seemed important to identify the genes and gene products involved in these mechanisms, as well as their respective functions. Thus, the transcriptome of *A. molluscorum* Av27 was sequenced using second generation technologies.

5.1 RNA-Sequencing

Transcriptome analysis of non-model organisms used to involve construction of a cDNA library, repeated rounds of normalization/subtraction and Sanger sequencing. cDNA microarrays were also commonly used for transcriptome analysis (Whitfield, Band et al. 2002; Mita, Morimyo et al. 2003; Paschall, Oleksiak et al. 2004; Papanicolaou, Joron et al. 2005; Beldade, Rudd et al. 2006). However, these methods had some disadvantages, such as errors related to clone mishandling and the fact that some transcripts are unstable when cloned into bacteria, affecting the results and downstream analysis (Weber, Weber et al. 2007).

RNA-Sequencing (RNA-Seq) is a method that allows the study of the transcriptome, providing information about gene expression (Febrer, McLay et al. 2011). This is possible because cDNA is sequenced through second-generation sequencing technologies, such as

Illumina's Genome Analyzer, Applied Biosystem's SOLiD, and Roche-454 GS FLX platforms (Febrer, McLay et al. 2011; Mäder, Nicolas et al. 2011). The use of these platforms reduces the costs and laborious analysis of the transcriptome, overcoming the problems associated with the previous techniques (Weber, Weber et al. 2007).

RNA-Seq is a useful method that can help improving the knowledge about bacterial gene regulation and expression. Many bacterial genomes are actually being re-annotated based on the information provided by RNA-Seq, and functions of previously unknown and/or unidentified genes are determined (Febrer, McLay et al. 2011). Transcriptomes of several eukaryotic and prokaryotic organisms have already been studied using this approach, revealing a high degree of concordance with established gene annotations (Liu, Livny et al. 2009; Oliver, Orsi et al. 2009; Jima, Zhang et al. 2010). This approach is particularly useful when there is no prior genomics information available on the species in study and when budgets are limited, since only the expressed parts of the genome are sequenced (Emrich, Barbazuk et al. 2007; Vera, Wheat et al. 2008; Ekblom, Slate et al. 2012).

RNA-Seq can also be used to study differential expression (changes in transcript abundance related to different conditions); this application is generally of major interest, since it helps to better understand gene function, development, phenotypic plasticity, local adaptation and speciation (Barakat, DiLoreto et al. 2009; Kristiansson, Asker et al. 2009; Wolf, Bayer et al. 2010; Mäder, Nicolas et al. 2011; Ekblom, Slate et al. 2012). The gene expression can be estimated through the number of reads of a particular gene generated by the cDNA sequencing (RNA-Seq), by analysing the available databases of expressed sequence tags (ESTs) for the genes of interest or by performing microarrays studies (Murray, Doran et al. 2007; Hoen, Ariyurek et al. 2008; Ekblom, Balakrishnan et al. 2010). This type of study usually requires a normalization step and the definition of the criteria that differentiate significant changes from those expected by chance alone (Mäder, Nicolas et al. 2011).

In Figure 3, a flow diagram is presented, showing the major steps of RNA-Seq when applied to bacterial transcriptomes. After extraction of high quality-RNA from the organism of interest, noncoding RNA (rRNA and tRNA) must be separated and discarded. Then, coding RNA is fragmented and a cDNA library is constructed. Finally, the library is sequenced using a second-generation sequencing platform. The sequence is then analysed using *in silico* tools (Febrer, McLay et al. 2011; Mäder, Nicolas et al. 2011). If the genome

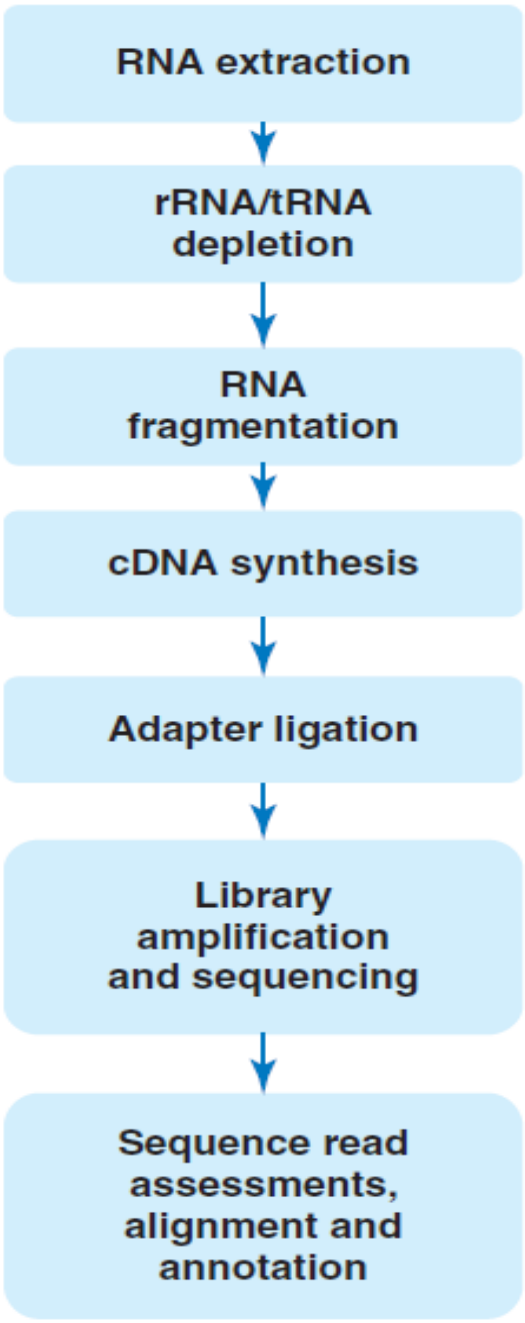


Figure 3 - Strategies used in RNA-Seq experiments for assessing different elements of the bacterial transcriptome. Adapted from Febrer, McLay et al. (2011).

of the organism under study has already been sequenced, the reads are mapped to the reference genome (Pinto, Melo-Barbosa et al. 2011). When the genome of the specie has not been sequenced yet, the annotation of the whole-transcriptome sequence datasets and the identification of the specific genes of interest can be achieved using data from related species (genomic reference species) (Ekblom, Slate et al. 2012). This process (new transcript annotation) requires substantial pipeline and/or manual post processing, essentially when the

experiment involves comparison between different conditions. In this case, one of the conditions can be taken as a reference and the transcribed regions can be subdivided according to other data sets (Güell, Noort et al. 2009; Xu, Wei et al. 2009; Mäder, Nicolas et al. 2011).

RNA-Seq presents some advantages: high reproducibility, high sensitivity, almost no noise and no prior annotation is required (Febrer, McLay et al. 2011; Mäder, Nicolas et al. 2011; Pinto, Melo-Barbosa et al. 2011). The use of second-generation sequencing technology is also advantageous, since the elimination of the cloning step minimizes errors and simplifies sample preparation. Besides, this method provides good coverage and allows characterization of the entire transcriptome of an organism, discovering new transcripts, identifying mutations, deletions, insertions and splicing alternatives and providing information about gene expression levels (Wang, Gerstein et al. 2009; Pinto, Melo-Barbosa et al. 2011; Park, Park et al. 2012).

Nevertheless, there are some problems associated with the use of this technology. For instance, the size of the transcripts can influence its detection (larger transcripts are detected more easily than small ones). Furthermore, there are some RNA-Seq steps that can introduce biases, such as fragmentation, synthesis of cDNA and mRNA enrichment (this last step is not always applied). The amount of information generated can also be considered a disadvantage, since it requires robust analysis, involving high bioinformatics knowledge and the use of powerful servers (Pinto, Melo-Barbosa et al. 2011).

5.2 cDNA library construction

Within a cell, the expressed genes are represented by the mRNA. Hence, the analysis of the transcriptome must be performed using the information contained in that mRNA. However, since RNA is a single-stranded molecule, it cannot be cloned directly. The solution is to use a specialized enzyme, the reverse transcriptase, to produce double-stranded (ds) cDNA, which can then be cloned. Using this cDNA, it is possible to construct libraries representing the set of transcripts of a cell, tissue or organism (Nagaraj, Gasser et al. 2007).

There are several methods for cDNA libraries construction; however, the conventional methods present several limitations. For instance, the synthesis of full-length cDNA clones is a major obstacle, especially for larger mRNAs (longer than 2 Kb) (Zhu,

Machleder et al. 2001). Besides, since the construction of the library usually involves the use of adaptors, it leads to libraries with up to 20% of undesirable ligation by-products (chimeras), as well as inserts derived from non-mRNA origins (genomic or mitochondrial DNA, ribosomal RNA, adaptor dimers) (Sambrook, Fritsch et al. 1989; Sudo, Chinen et al. 1994). The fact that these methods rely on methylation is also a constraint, since the methylation process is inefficient for cloning and does not provide complete protection for the internal restriction sites (McClelland, Nelson et al. 1994). Beyond these limitations, the conventional cDNA library construction methods involve complicated, multi-step manipulations of mRNA and cDNA intermediates, increasing the risk for mRNA or cDNA-RNA duplexes degradation (Zhu, Machleder et al. 2001).

The SMART™ (Switching Mechanism at the 5' end of the RNA Transcript) approach has been recently used to construct cDNA libraries (Pascoal, Carvalho et al. 2012; Sadr-Shirazi, Shayan et al. 2012; Zhou, Zhang et al. 2012). This method takes advantage of two intrinsic properties of Moloney murine leukemia virus (MMLV) reverse transcriptase: reverse transcription and template switching (Zhu, Machleder et al. 2001). The simultaneous employment of these two properties allows the synthesis of cDNA clones with greater length and directional cloning. In this method, both ends of the first-strand cDNA are anchored through the addition of a distinct SfiI site to each end during reverse transcription:

- 5'end: SfiIB restriction site on a modified oligo(dT) primer
- 3'end: SfiIA restriction site on a template-switching oligonucleotide that serves as an extended template when the reverse transcriptase reaches the end of the RNA molecule (Zhu, Machleder et al. 2001).

The template-switching phenomenon presents higher efficiency when the reverse transcriptase has reached the end of the RNA template (Chenchik, Zhu et al. 1998). Hence, the prematurely terminated cDNAs are eliminated during the cloning process, since they lack the SfiIA restriction site (Zhu, Machleder et al. 2001).

This process ensures that the constructed cDNA libraries present higher yields of representative, full-length clones (even when the starting concentration of RNA is low) and a higher proportion of clones with intact ORFs (about 77%, which is 2-3 times higher than the proportion obtained with the conventional methods). Besides, the SMART™ approach is simpler and faster than other full-length cDNA library construction methods (Zhu, Machleder et al. 2001).

5.3 Normalization

Normalization involves the construction of a depletion library. This process consists on the elimination of non-coding RNAs and leveling of the concentrations of the different transcripts, in order to reduce the frequency of highly expressed genes.

Normalization is useful because more than 95% of the total RNA in a cell is constituted by the rRNA/tRNA fraction, which makes it harder to analyse the useful transcripts (Mäder, Nicolas et al. 2011). Besides, the cellular mRNA mass is usually constituted by:

- 20 % of 10-20 abundant genes (several thousand mRNA copies per cell);
- 40-60 % of several hundred genes of medium abundance (several hundred mRNA copies per cell);
- 20-40% of several thousand rare genes (<10 mRNA copies per cell) (Carninci, Shibata et al. 2000).

This uneven distribution makes the discovery and analysis of rare genes very difficult and inefficient (Zhulidov, Bogdanova et al. 2004). Besides, without the normalization step some transcripts would be redundant and analysed several times, leading to a waste of time and money (Bogdanova, Shagina et al. 2009; Ekblom, Slate et al. 2012).

The distinction between mRNA and other noncoding RNAs is usually made using the polyA tail. However, contrary to what is observed in eukaryotes, bacteria do not present polyadenylated RNA. Hence, only recently it was reported the application of RNA-Seq to bacterial genomes (Febrer, McLay et al. 2011). An approach to overcome this problem is the introduction of a mRNA enrichment step (Yoder-Himes, Chain et al. 2009). However, other methods can be applied in prokaryotes, such as hybridization capture of rRNAs by antisense oligonucleotides followed by pull down through binding to magnetic beads and degradation of processed RNAs such as mature rRNAs and tRNAs by a 5'–3' exonuclease that specifically digests RNA species with a 5'-monophosphate end (Mäder, Nicolas et al. 2011).

The depletion library can be constructed using a duplex-specific nuclease (DSN) isolated from the Kamchatka crab (*Paralithodes camtschaticus*). This nuclease is thermostable and specific for double-stranded DNA or DNA-RNA hybrids (Shagin, Rebrikov et al. 2002; Zhulidov, Bogdanova et al. 2004; Bogdanova, Shagina et al. 2009). DSN-depletion is the most common normalization method, since it is simple and allows the

efficient removal of several known sequences prior to library cloning, thus eliminating the need for laborious physical separation (Zhulidov, Bogdanova et al. 2004; Bogdanova, Shagina et al. 2009; Ekblom, Slate et al. 2012). Besides, this method is also efficient when full-length-enriched cDNA (prepared from total RNA) is used, unlike the previous techniques that involved separation of single-stranded and double-stranded cDNA using hydroxylapatite columns or magnetic beads, digestion of the double-stranded cDNA by restriction endonucleases and amplification of single-stranded cDNA using suppression PCR (Zhulidov, Bogdanova et al. 2004; Bogdanova, Shagina et al. 2009).

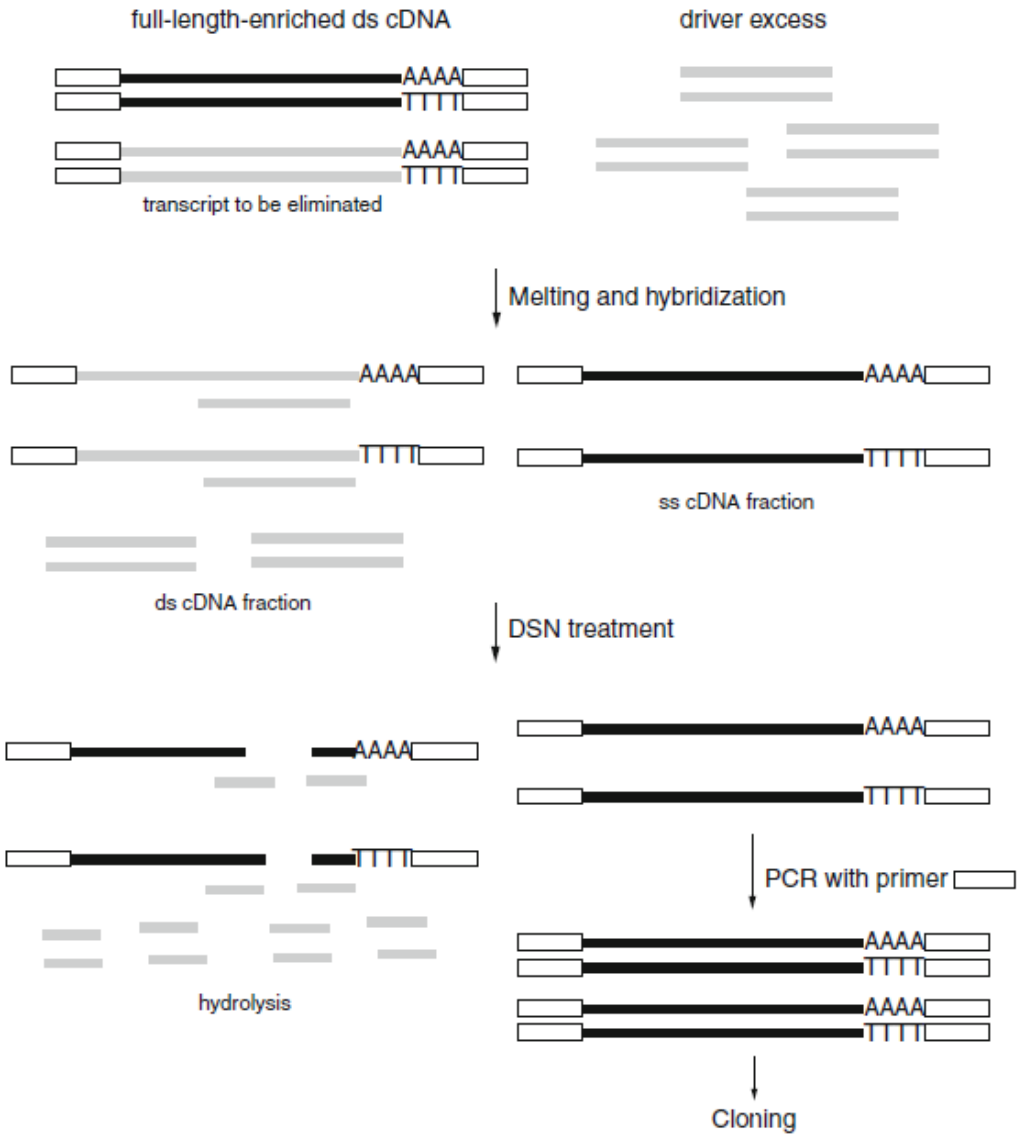


Figure 4 - Schematic representation of the DSN depletion method applied to cDNA.
Black line: transcript of interest; gray line: transcripts to be eliminated (Bogdanova, Shagina et al. 2009).

As can be seen in Figure 4, the first step of the DSN depletion method involves the mixture of the double stranded cDNA with driver DNA, which represents the fragments of genes to be eliminated. Since DSN requires a perfect DNA-DNA duplex of at least 10 bp in length, the driver DNA must respect that minimum size; besides, it must be provided in excess. The next step is denaturation, followed by hybridization between the driver fragments and the sequences to be eliminated. The result is that the transcripts of interest become single-stranded cDNA, while the fragments to be removed remain as double-stranded cDNA, being hydrolyzed by DSN (Bogdanova, Shagina et al. 2009). Since the DSN depletion method is based on the second-order solution hybridization kinetics, the depletion process is accompanied by cDNA normalization (Young and Anderson 1985). Highly abundant transcripts have time to pass into the ds form, so they become substrate for DSN (Zhulidov, Bogdanova et al. 2004; Bogdanova, Shagina et al. 2009).

RNA-Seq studies involving gene expression analysis require that the sequenced reads' relative abundance is as little biased as possible. Hence, these studies mostly use unnormalised cDNA libraries. However, it has been demonstrated that there is a positive correlation between the expression levels on both normalised and unnormalised data, which confirms the validity of the use of normalised cDNA libraries in gene expression analysis. This is possible because the cDNA levels present enough variation even after normalization (Ekblom, Slate et al. 2012). In fact, several authors report the application of cDNA normalization when studying the differential expression of some genes through the RNA-Seq method (Kristiansson, Asker et al. 2009; Schwarz, Robertson et al. 2009; Ekblom, Slate et al. 2012).

5.4 Second-generation technologies

DNA sequencing used to be performed by the Sanger method, which was expensive, time consuming and laborious. Nowadays, second-generation sequencing technologies are preferred, since they allow massive parallel sequencing of the whole genome, are cheaper, and do not require large automated facilities (Hudson 2007; Metzker 2010). Hence, sequencing of genomes and transcriptomes is now easier and more frequent. There are several second generation sequencing methods developed by different companies (Solexa, Illumina, SOLiD), but all of them use nanotechnology and generate hundreds of thousands

of small sequence reads at one time (Hudson 2007; Metzker 2010; Febrer, McLay et al. 2011).

454 Life Sciences (Roche) developed a sequencing technology based on pyrosequencing (Hudson 2007). In this method, the base incorporation is detected in real time by pyrophosphate release (Ronaghi, Karamohamed et al. 1996; Ronaghi, Uhlén et al. 1998). Hence, no gels or capillaries are required to separate the extension products by size, as in the Sanger method (Hudson 2007). In the Sanger method, DNA amplification required sub-cloning in bacteria (Sanger, Nicklen et al. 1977). However, in the 454 method this step is not required, since the sheared DNA fragments are linked to beads and emulsion-phase PCR is used to amplify those fragments (Hudson 2007). This emulsion-based method (Figure 5) involves several steps: (1) random fragmentation of the entire genome; (2) addition of specialized common adapters to the fragments and immobilization of each individual fragment in its own bead through adapter sequence complementarity; (3) capturing of each bead in an emulsion droplet and amplification of the individual fragment (emulsion-phase PCR) (Margulies, Egholm et al. 2005).

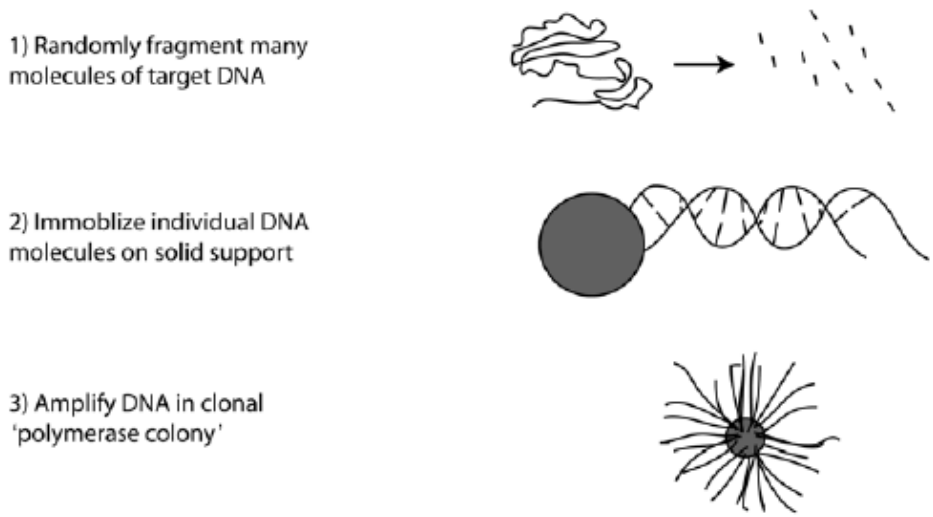


Figure 5 - DNA amplification through emulsion-phase PCR. Adapted from Hudson (2007).

Then, the beads containing the amplified DNA are immobilized in a picolitre-sized well plate containing 1.6 million wells, where a polymerase-mediated elongation occurs (Figure 6) (Margulies, Egholm et al. 2005; Hudson 2007). The presence of the template-containing beads in each well is detected through the presence of a known four-nucleotide sequence at the beginning of the reads (Margulies, Egholm et al. 2005). In this process, a

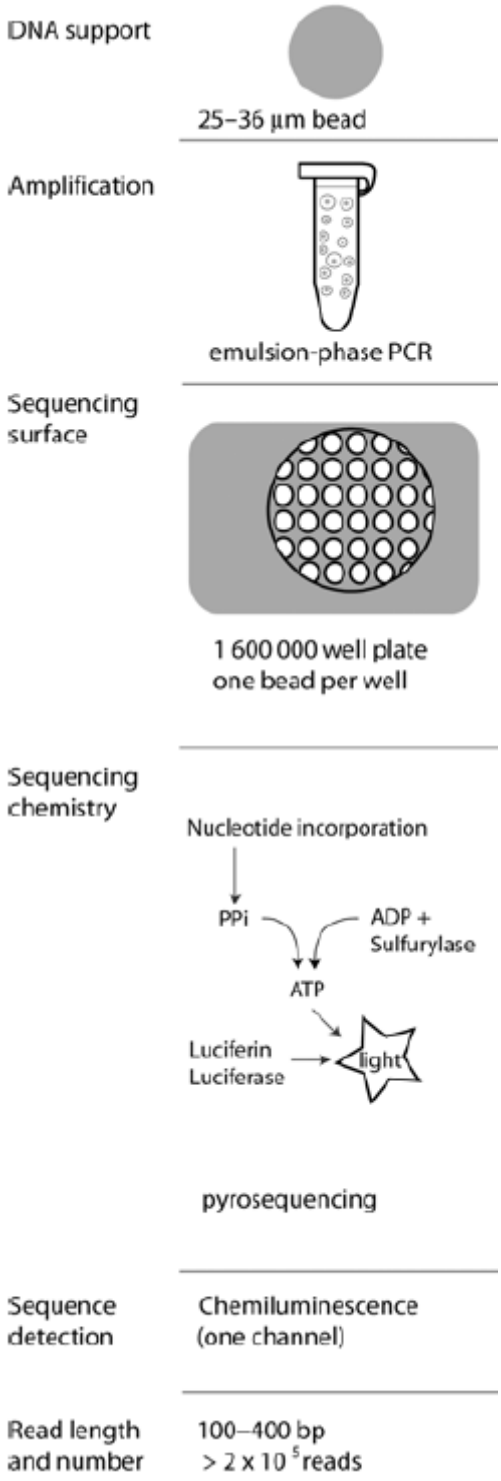


Figure 6- Schematic representation of the Roche 454 pyrosequencing method. Adapted from Hudson (2007).

flow of nucleotides is passed through the plate (each nucleotide at a time) followed by a nuclease-containing wash to ensure that no nucleotide remains in any well before the next nucleotide flow. When a nucleotide is incorporated, it triggers the release of pyrophosphate

(PPi) and the generation of photons, producing a flash of chemiluminescence (Margulies, Egholm et al. 2005). After the sequencing of the fragments, the sequences must be aligned. In this method, the optimal alignment is determined by using the signal strengths at each nucleotide flow instead of individual base calls. The high oversampling obtained with the 454 technology increases the quality of the consensus sequence (Margulies, Egholm et al. 2005).

The 454 sequences are distributed evenly across the cDNA of a given gene, which helps to obtain blast alignments (Weber, Weber et al. 2007; Vera, Wheat et al. 2008). However, it also results in multiple fragments per gene, demanding additional assessment in downstream analyses in order to discover the relation between these fragments. Besides, since the 454 sequences are derived from both the cDNA strands, the directional orientation of the sequencing data is unknown. Nevertheless, blast annotation can be used to infer about this directionality (Vera, Wheat et al. 2008).

In summary, 454 pyrosequencing is a faster, cheaper and easier process that also increases sequencing depth and coverage, being the most common sequencing method when *de novo* characterization of transcriptomes of non-model organisms is intended (Ekblom, Slate et al. 2012). The preference for the 454 pyrosequencing in these studies derives from the fact that this sequencer generates long reads that provide valuable information even when a reference genome is not known (Novaes, Drost et al. 2008; Vera, Wheat et al. 2008).

Nevertheless, there are some problems associated with the transcriptome analysis of organisms that lack a fully-sequenced genome, since it may be difficult to assess the number of genes expressed. This happens for various reasons:

- some contigs remain as separate sequences after contig-joining, since they lack a match in public databases;
- some fragments that would be expected to lack a match in public databases may match with conserved regions in known genes or even poorly-conserved regions such as un-translated regions;
- some fragments may be too short, thus not being able to allow statistically meaningful matches;
- a single sequencing run does not provide a sufficient level of coverage to allow the production of complete transcript sequences by sequence assembly alone (Meyer, Aglyamova et al. 2009).

Solving these problems is of great interest, since the quantification of gene expression is one of the primary questions for transcriptome sequencing (Meyer, Aglyamova et al. 2009).

5.5 Expressed Sequence Tags

As previously mentioned, the RNA-Seq method involves the construction of cDNA libraries followed by sequencing of the cDNA clones (randomly, from both directions, in a single-pass run). The result of this sequencing step is a set of expressed sequence tags (ESTs). Hence, ESTs can be described as short (100-800 bp) unedited copies of the mRNA itself (Nagaraj, Gasser et al. 2007).

The traditional EST sequencing method involves the anchoring of each sequence at the 3' or 5' end of the transcript. A different approach is the use of second-generation technologies to sequence ESTs. This method, also known as transcriptome sequencing, consists in the sequencing of entire, random mRNA fragments and posterior computational assembly (Hudson 2007).

Due to the fact that these short sequences are only sequenced once, they are highly susceptible to errors, especially at the ends (the quality of the sequence is significantly higher in the middle). Another problem related to EST data is the fact that they can be generated through various different protocols, leading to redundancy and under- or over-representation of selected transcripts (Nagaraj, Gasser et al. 2007).

ESTs are very useful, as they enable gene discovery, complement genome annotation, aid gene structure identification, establish the viability of alternative transcripts, guide single nucleotide polymorphism (SNP) characterization and facilitate proteome analysis (Jongeneel 2000; Rudd 2003; Qunfeng, Lori et al. 2005). This is possible because EST sequences contain information only about coding DNA (lacking introns and intragenic regions), which facilitates data interpretation (Bouck and Vision 2007; Parchman, Geist et al. 2010).

6. Quantitative Real-Time PCR

When expression level studies are performed, the data resulting from the *in silico* analyses require posterior validation to confirm that the genes are in fact differentially expressed. Some of the methods that can be used to validate the data are the reverse-

transcriptase polymerase chain reaction (RT-PCR), the quantitative real-time polymerase chain reaction (qPCR) or β-galactosidase assays (Yoder-Himes, Chain et al. 2009; Isabella and Clark 2011; Park, Park et al. 2012).

Quantification methods are very useful, as they allow evaluation of gene copy number and mRNA expression, diagnosis of infectious diseases, investigation of the efficiency of gene delivery systems and verification of biological product purity (Lie and Petropoulos 1998). The biological response to various stimuli can also be monitored through the measurement of gene expression (Tan, Sun et al. 1994; Huang, Xiao et al. 1995; Prud'homme, Kono et al. 1995).

Polymerase chain reaction (PCR) usually provides reaction products that can be separated through electrophoresis and analysed by comparison of band intensities. However, since this approach is not quantitative, it only allows an estimation of the relative starting concentrations (Lie and Petropoulos 1998).

On the other hand, quantitative real-time PCR (qPCR) allows quantification of expression through the use of fluorescent DNA-binding dyes. The fluorescence of these dyes is detected when they are bound to double-stranded DNA and it is proportional to the DNA concentration (Figure 7). During amplification, the fluorescence level will rise above a defined baseline (Invitrogen 2008). The amplification cycle at which this happens is known as C_T (threshold cycle), and this value is what allows the quantification: the higher the concentration of the target gene, the lower the number of cycles needed to raise the emission intensity above the pre-defined baseline (Heid, Stevens et al. 1996; Lie and Petropoulos 1998).

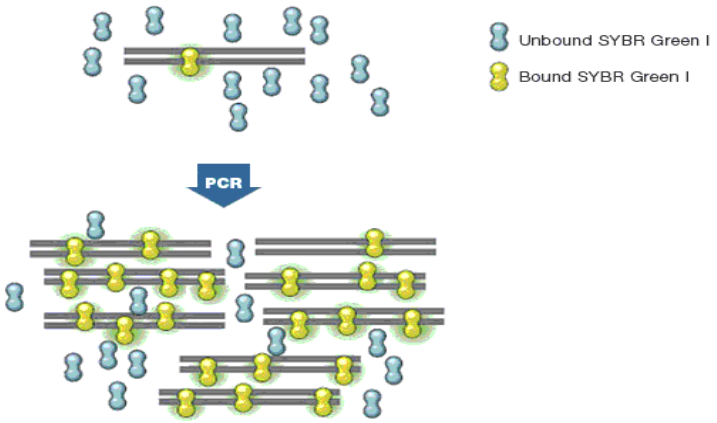


Figure 7 - Schematic representation of the use of fluorescent DNA-binding dyes in real-time PCR (http://www.bio-rad.com/webroot/web/images/lsr/solutions/technologies/gene_expression/pcr/technology_detail/gxt28_img1.gif).

The "real-time" designation originates from the fact that detection occurs during each PCR cycle; the electrophoresis step is therefore unnecessary (Lie and Petropoulos 1998).

The lack of post-PCR sample handling is advantageous, since the results are obtained faster, a higher throughput assay is achieved and possible contaminations are prevented. This method is also accurate and has a very large dynamic range of starting target molecule determination (Heid, Stevens et al. 1996).

Depending on the objective of the experiment, the quantification can be absolute or relative. Absolute quantification is the most adequate when the exact number of transcript copies is required, since it usually relates the PCR signal to a standard curve. However, in some circumstances, the determination of the absolute transcript copy number may not be necessary. In these situations, relative quantification can be used. This relative method evaluates the change in the expression of the target gene in comparison with some reference group (untreated control, for example) (Livak and Schmittgen 2001).

7. Objectives

TBT is a toxic organotin compound that affects mainly aquatic environments. Despite the legislation that limits the use of TBT, its high persistence and the fact that it is still used in some countries makes TBT pollution a problem of concern.

The molecular mechanisms of TBT resistance in various organisms have been studied by some authors, but little is known about these mechanisms, mainly because it seems to be different in the different organisms. Likewise, little is known about the TBT biodegradation. The full comprehension of the resistance and degradation mechanisms may allow the use of organisms to decontaminate polluted sites.

In this study, a transcriptome approach was employed in order to identify genes involved in TBT resistance and degradation. To that end, the transcriptome of *Aeromonas molluscorum* Av27 was sequenced by the RNA-Seq method. Furthermore, quantitative real-time PCR was performed to validate the results from the transcriptome analysis.

This approach provides information about the genes that are differentially expressed in the presence of TBT. Furthermore, the study of the function of those genes might shed more light on the TBT resistance and degradation mechanisms in *Aeromonas molluscorum* Av27. The identified differentially expressed genes can be further investigated for their application to develop bioreporters for TBT.

MATERIALS AND METHODS

8. Transcriptome analysis

This study was started from the analysis of the results of the transcriptome. But, even so, the materials and methods used to obtain these results are also included for a better comprehension of all the steps involved and required prior to the transcriptome analysis.

The main goal of the present thesis consisted in analyzing the transcriptome sequencing data followed by validation of the results by quantitative real-time PCR.

8.1 Cellular growth

Aeromonas molluscorum Av27 cells were grown, with or without TBTCl (97%) (Fluka), in Marine Broth medium at 26 ºC, 180 rpm, in the dark (since TBT can be photo degraded). Hereafter, TBTCl will be referred to as TBT.

The following conditions were tested:

- control (cells not exposed to TBT);
- 5 μM TBT: environmentally relevant concentration (Antizar-Ladislao 2008);
- 50 μM TBT: concentration at which *Aeromonas molluscorum* Av27 is known to degrade TBT (Cruz, Caetano et al. 2007).

The erlenmeyers (250 mL) used for cell growth were previously immersed in hydrochloric acid (10 %) for at least 24 hours and then washed with distilled water, to remove any organic residue.

Cell growth was monitored by a change in optical density (OD) at 600 nm (Genesys 20 Visible Spectrophotometer, Thermo Scientific). When the cultures reached the desired OD ($A_{600\ nm}$ = 0.5, exponential growth phase), the cells were precipitated by centrifugation and the pellets were kept at -80ºC.

8.2 RNA extraction and purification

RNA was extracted using TRIzol Max Bacterial Isolation Kit (Invitrogen) according to manufacturer's instructions, and purified with the Turbo DNA-free Kit (Ambion). The protocols are described below in detail.

RNA Extraction - TRIzol® Max™ Bacterial Isolation Kit (Invitrogen)

1. Transfer 1.5 mL of bacterial culture (up to 1 × 10^8 cells) to a pre-chilled microcentrifuge tube.
2. Centrifuge the tube at 6000 × g for 3 minutes at 4°C in a microcentrifuge.
3. Pre-heat 200 µL Max Bacterial Enhancement Reagent to 95°C.
4. After centrifugation, decant the supernatant and resuspend the cell pellet in pre-heated 200 µL Max Bacterial Enhancement Reagent from the previous step. Mix well by pipetting up and down.
5. Incubate the tube at 95°C for 4 minutes.
6. Add 1 mL TRIzol® Reagent to the lysate and mix well.
7. Incubate the tube at room temperature for 5 minutes.
8. Add 0.2 mL cold chloroform and mix by shaking the tube vigorously by hand for 15 seconds.
9. Incubate the tube at room temperature for 2–3 minutes.
10. Centrifuge the samples at 12,000 × g for 15 minutes at 4°C. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless aqueous phase containing RNA.
11. Transfer ~400 µL of the colorless upper phase containing RNA to a fresh tube.
12. Add 0.5 mL cold isopropanol to the aqueous phase to precipitate RNA. Mix by inverting the tube.
13. Incubate the tube at room temperature for 15 minutes.
14. Centrifuge at 15,000 × g for 10 minutes at 4°C.
15. Remove the supernatant carefully without disturbing the RNA pellet (a gel-like pellet formed at the side and bottom of the tube).
16. Resuspend the pellet in 1 mL 75% ethanol. Mix well by vortexing.
17. Centrifuge at 7500 × g for 5 minutes at 4°C. Discard the supernatant.
18. Air-dry the RNA pellet. Do not dry the RNA pellet by centrifugation under vacuum.
19. Resuspend the RNA pellet in 50 µL RNase-free water by pipetting up and down, and incubating for 10 minutes at 60°C, if needed.

RNA Purification - TURBO DNA-free™ Kit (Ambion)

1. Add 5 µL 10x TURBO DNase Buffer and 1 µL TURBO DNase to the RNA and mix gently.
2. Incubate at 37ºC for 45 minutes.
3. Add 5.5 µL resuspended DNase Inactivation Reagent and mix well.
4. Incubate 2 minutes at room temperature, mixing occasionally.
5. Centrifuge at 10,000 × g for 1 minute and 30 seconds.
6. Transfer the supernatant, which contains the RNA, into a fresh tube.

DNA contamination was confirmed by PCR amplification of the 16S rRNA gene. The thermal cycler used was the MJ Mini (Bio-Rad) and the reagents were provided by Promega. The primers used in this procedure were provided by ThermoElectron® and are shown in Table 1.

Table 1 - Primers used to amplify the 16S rRNA gene.

	Sequence
16SFw	5'-AGAGTTTGATCCTGGTCAG-3'
16SRv	5'-AAGGAGGTGATCCAGCC-3'

The reagents and amounts used in the preparation of the amplification reaction are listed in Table 2. The PCR program used is shown in Table 3. Total DNA of Av27 strain was used as positive control.

Table 2 - Components and respective volumes used in the amplification reaction.

Component	**Volume/Reaction (µL)**
$MgCl_2$ (25 mM)	1.5
Buffer (5 ×)	2.5
dNTP mix (10 mM)	0.25
DMSO	0.63
16SFw (10 pmol/µL)	0.38
16SRv (10 pmol/µL)	0.38
Taq Pol (5 U/µL)	0.06
RNA/total DNA	1
dH_2O	6.8
TOTAL	13.5

Table 3 - PCR program used for amplification of the 16S rRNA.

	Temperature (ºC)	Time (minutes)	Nº Cycles
Initialization	94	5	1
Denaturation	94	0.5	30
Annealing	56	0.5	
Extension/elongation	72	1.5	
Final elongation	72	10	1

RNA concentration was determined using Qubit (Invitrogen) as described below, and also using Nanodrop (Thermo Scientific) according to manufacturer's instructions.

RNA Quantification - Qubit™ (Invitrogen)

1. Set up the number of 0.5 mL tubes needed for standards and samples. The Qubit™ RNA assay requires 2 standards.

   Note: Use only thin-wall, clear 0.5 mL optical-grade real-time PCR tubes.
2. Make the Qubit™ working solution by diluting the Qubit™ RNA reagent 1:200 in Qubit™ RNA buffer. Use a clean plastic tube. Do not mix the working solution in a glass container.

   Note: The final volume in each tube must be 200 µL. Each standard tube will require 190 µL of Qubit™ working solution, and each sample tube will require anywhere from 180 µL to 199 µL.
3. Load 190 µL of Qubit™ working solution into each of the tubes used for standards.
4. Add 10 µL of each Qubit™ standard to the appropriate tube and mix by vortexing 2–3 seconds, being careful not to create bubbles.

   Note: Careful pipetting is critical to ensure that exactly 10 µL of each Qubit™ RNA standard is added to 190 µL of Qubit™ working solution. It is also important to label the lid of each standard tube correctly as calibration of the Qubit® 2.0 Fluorometer requires that the standards be introduced to the instrument in the right order.
5. Load Qubit™ working solution into individual assay tubes so that the final volume in each tube after adding sample is 200 µL.

Note: The sample can be anywhere between 1 μL and 20 μL, therefore, load each assay tube with a volume of Qubit™ working solution anywhere between 180 μL and 199 μL.

6. Add each sample to assay tubes containing the correct volume of Qubit™ working solution (prepared in step 5) and mix by vortexing 2–3 seconds. The final volume in each tube should be 200 μL.
7. Allow all tubes to incubate at room temperature for 2 minutes.
8. Read samples on the Qubit® Fluorometer.

8.3 cDNA library construction and normalization

The total RNA obtained (≈3 μg) was sent to Evrogen company (Russia Federation), where cDNA was synthetized using the SMART approach (Zhu, Machleder et al. 2001).

As mentioned in section 5.1, transcriptome analysis studies usually require a normalization step. Hence, a duplex-specific nuclease (DSN) normalization method (Zhulidov, Bogdanova et al. 2004) was performed also by Evrogen.

8.4 Transcriptome sequencing and data annotation

The transcriptomes were sequenced and annotated by Biocant (Portugal).

Pyrosequencing was performed with 454 GS FLX Titanium. Then, 454 reads were trimmed for removal of low quality parts, reads with less than 100 nucleotides and low complex areas, as well as ribosomal, mitochondrial and chloroplast reads. The remaining reads were assembled into contigs using 454 Newbler 2.6.

To identify the translations frame, the contigs were BLASTx against Swissprot using an e-value threshold of e-value $\leq 10^{-6}$. An internal algorithm was used to translate the regions. The contigs without previous translation were run through FrameDP (Gouzy, Carrere et al. 2009), a software used to identify putative peptides; the remaining contigs without translation were finally run by ESTScan (Lottaz, Iseli et al. 2003). The resulting putative proteins were annotated using the BLASTp against the nr@ncbi database. Functional annotation and Gene Ontology (GO) identification were obtained using the InterproScan (EMBL-EBI 2013).

To identify the differential expression, the putative proteins were first clustered using a CD-Hit 454 application (Niu, Fu et al. 2010) (90% similarity) to eliminate redundant sequences. Then, contigs encoding non-redundant proteins were used as reference to map the reads; 454 Newbler Mapping 2.6 was used in the mapping process. These steps allowed the quantification of the number of reads that mapped in the references formed by the contigs. The application MyRNA (Langmead, Hansen et al. 2010) was used to obtain the p-value, from which it is possible to get a statistical evidence of the differential protein expression levels.

These procedures are summarized in Figure 8 and Figure 9.

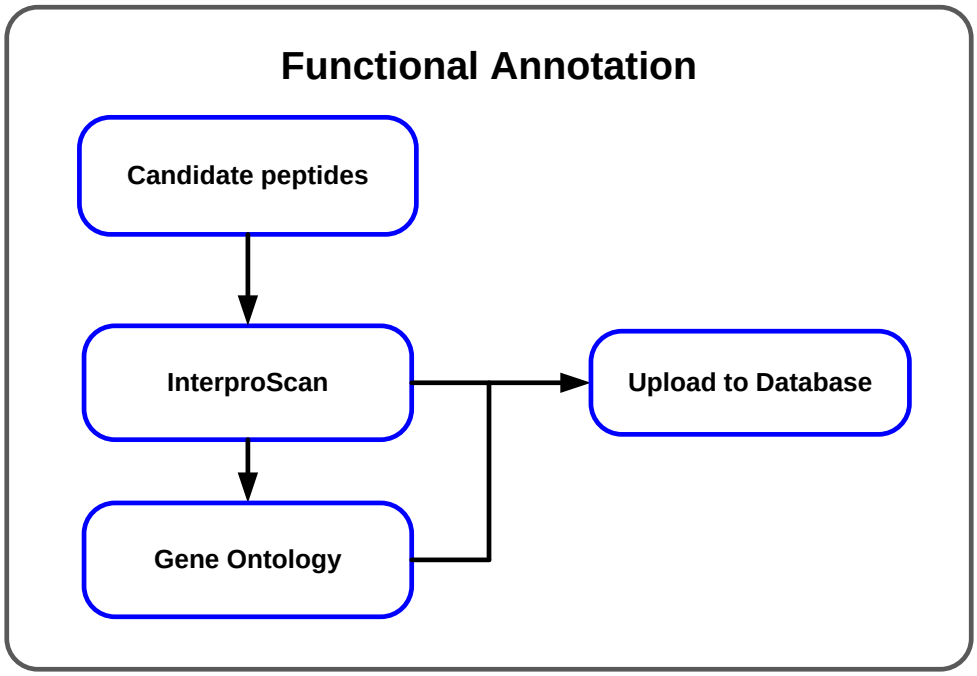


Figure 8 - Schematic representation of the procedure followed to obtain functional annotation (provided by Biocant).

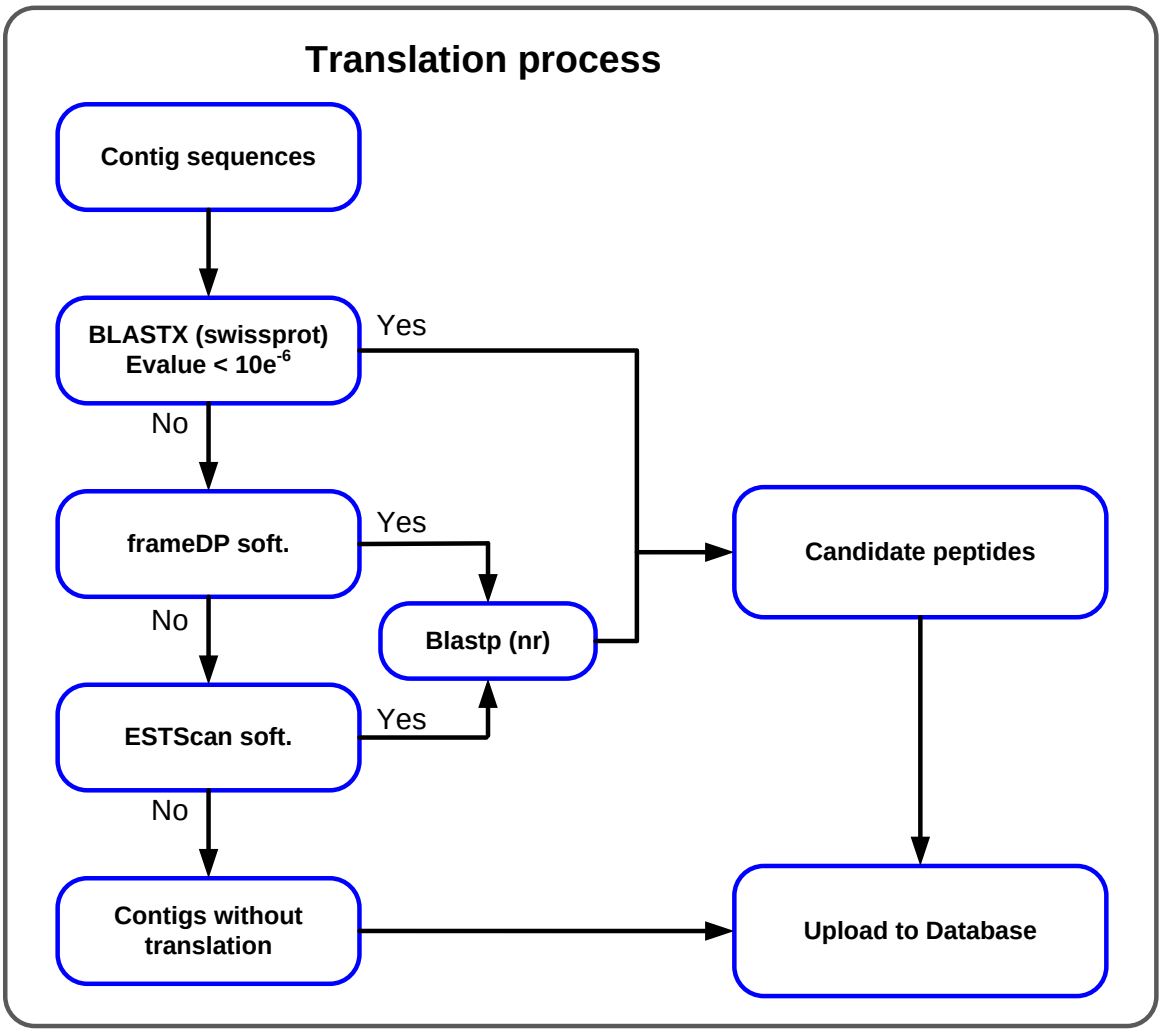


Figure 9 - Schematic representation of the procedure followed to identify the translation frame annotation (provided by Biocant).

8.5 Differential expression analysis

The transcriptome of *A. molluscorum* Av27 was analysed with the purpose of identifying genes involved in TBT resistance and degradation. This analysis involved the identification of over-expressed (expression ratio ≥ 2) and under-expressed (expression ratio < 0.5) genes in the transcriptome and the study of the respective gene ontology.

Previous studies reported the identification of pyoverdine as the peptide responsible for triphenyltin (TPT) degradation (Inoue, Takimura et al. 2000; Inoue, Takimura et al. 2003). Hence, this peptide or others with similar function were investigated in the transcriptome of strain Av27.

The transcriptome was also screened for genes involved in metal and antibiotic resistance, which might be related to TBT resistance as suggested by Suzuki, Fukagawa et al. (1992) and Jude, Arpin et al. (2004). Besides, since *A. molluscorum* Av27 cells aggregate in the presence of TBT, aggregation proteins were also sought (Cruz, Oliveira et al. 2010).

Moreover, other genes described by other authors as being involved in TBT resistance were also investigated in Av27's transcriptome as, for instance, *sug*E (Cruz, Micaelo et al. 2013) and *aheABC* (Hernould, Gagné et al. 2008). The results obtained in this study were also compared with those reported by Dubey (2006), regarding the analysis of the transcriptome of *Pseudomonas aeruginosa* 25W, a TBT resistant strain.

9. Use of qPCR to validate the transcriptome results

9.1 Cellular growth

Aeromonas molluscorum Av27 cells were grown in the same conditions used for transcriptome analysis (section 8.1). Briefly, the cells were grown in Marine Broth medium at 26 ºC, 180 rpm, to an optical density ($A_{600\ nm}$) of 0.5 (exponential growth phase). Two conditions were tested (exposure to 5 and 50 μM of TBT) in addition to the control condition. A triplicate of each condition was prepared.

9.2 RNA extraction and purification

RNA was extracted and purified with the Rneasy Mini Kit (Qiagen), according to manufacturer's instructions. The protocols are described below in detail.

RNA Extraction – RNeasy® Mini Kit (Qiagen)

1. Loosen the bacterial pellet by flicking the bottom of the tube.
   Note: do not use more than 10^9 bacteria.
2. Resuspend the bacteria thoroughly in 100 μL of lysozyme-containing TE buffer (Gram negative bacteria: 400 μg/mL) by vortexing. Incubate at room temperature for 5 minutes.

3. Add 350 µL Buffer RLT to the sample. Mix thoroughly by vortexing vigorously. If insoluble material is visible, centrifuge for 2 minutes at maximum speed, and use only the supernatant in subsequent steps.
   Note: Ensure that β-ME is added to Buffer RLT before use.
4. Add 250 µL ethanol (96–100%) to the lysate. Mix thoroughly by pipetting. Do not centrifuge. A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

RNA Purification – RNeasy® Mini Kit (Qiagen)

1. Apply the sample (usually 700 µL), including any precipitate that may have formed, to an RNeasy mini column placed in a 2 mL collection tube. Close the tube gently and centrifuge for 15 seconds at ≥8000 x g. Discard the flow-through. If the volume exceeds 700 µL, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.
2. Pipet 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥8000 x g to wash. Discard the flow-through.
3. Add 10 µL DNase I (~3 U/µL) to 8 µL DNase I Reactivation Buffer (10 x) and 62 µL RNase-free water. Mix by gently inverting the tube.
   Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.
4. Pipet the DNase I incubation mix (80 µL) directly onto the RNeasy silica-gel membrane, and place on the bench top (20–30°C) for 15 minutes.
   Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy column.
5. Pipet 350 µL Buffer RW1 into the RNeasy mini column, and centrifuge for 15 seconds at ≥8000 x g. Discard the flow-through.
6. Transfer the RNeasy column into a new 2 mL collection tube. Pipet 500 µL Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 seconds at ≥8000 x g to wash the column. Discard the flow-through.
   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.

7. Add another 500 μL Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 2 minutes at ≥8000 x g to dry the RNeasy silica-gel membrane. It is important to dry the RNeasy silica-gel membrane since residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

   Note: Following the centrifugation, remove the RNeasy mini column from the collection tube carefully so the column does not contact the flow-through as this will result in carryover of ethanol.
8. Place the RNeasy column in a new 2 mL collection tube, and discard the old collection tube with the flow-through. Centrifuge at full speed for 1 minute.
9. To elute, transfer the RNeasy column to a new 1.5 mL collection tube. Pipet 30–50 μL RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at ≥8000 x g to elute.
10. If the expected RNA yield is >30 μg, repeat the elution step (step 9) as described with a second volume of RNase-free water. Elute into the same collection tube. To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 9). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

DNA contamination was confirmed by PCR amplification of the 16S rRNA gene. The thermal cycler used was the MJ Mini (Bio-Rad) and the reagents were provided by Promega. The reagents, primers and PCR program were the same used in the transcriptome analysis protocol (section 8.2).

RNA concentration was determined using Nanodrop (NanoDrop® 2000 Spectrophotometer) according to manufacturer's instructions.

9.3 cDNA synthesis

Once RNA with the desired concentration and purity was obtained, cDNA was synthesized using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen).

cDNA synthesis - SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen)

1. Mix and briefly centrifuge each component before use.
2. For each reaction, combine the following in a sterile 0.5-mL tube:

Component	Volume (µL)
RNA (5 µg)	n
dNTP mix (10 mM)	1
Random hexamers (50 ng/µl)	1
RNase-free water	up to 10

3. Incubate the RNA/primer mixture at 65°C for 5 minutes, then place on ice for at least 1 minute.
4. In a separate tube, prepare the following reaction mix, adding each component in the indicated order.

Component	1 Reaction	10 Reactions
RT buffer (10X)	2 µL	20 µL
$MgCl_2$ (25 mM)	4 µL	40 µL
DTT (0.1 M)	2 µL	20 µL
RNaseOUT™ (40 U/µL)	1 µl	10 µL

5. Add 9 µL of the reaction mix to each RNA/primer mixture from step 2, mix gently, and collect by brief centrifugation.
6. Incubate at room temperature for 2 minutes.
7. Add 1 µL of SuperScript™ II RT to each tube. Minus RT Control: Add 1 µL RNase-free water instead of the RT.

   Note: The Minus RT Control allows to assess DNA contamination.
8. Incubate at room temperature for 10 minutes.
9. Incubate at 42°C for 50 minutes.
10. Terminate the reaction at 70°C for 15 minutes. Chill on ice.
11. Collect the reaction by brief centrifugation. Add 1 µL of RNase H to each tube and incubate for 20 minutes at 37°C. The reaction can be stored at -20°C or used for PCR immediately.
12. Assess cDNA quality by PCR amplification of the 16S rRNA, as described in section 8.2.

9.4 Quantitative Real-time PCR (qPCR)

Five reference genes and 14 target genes were selected to validate the transcriptome results.

Reference genes *gyr*B, *rpo*D and *rps*L were selected based on the bibliography search, while *opm*A and *rlm*L were chosen because their expression was found to be constant in transcriptome of *A. molluscorum* Av27 in all the conditions tested. *gyr*B and *rpo*D are referred as housekeeping genes of the genus *Aeromonas* and were thus selected (Soler, Yáñez et al. 2004). *rps*L is a reference gene commonly used in gene expression studies involving *Aeromonas hydrophila* (the reference species for the genus *Aeromonas*) (Lukkana, Wongtavatchai et al. 2011). In addition to these, the reference genes *opm*A and *rlm*L were also used due to their constant expression in the three conditions analysed. The primers used to amplify the reference genes are presented in Table 4.

Table 4 - Primers used to amplify each reference gene, with respective melting temperature and product size.

Gene	Primer (5'-3')	Tm (ºC)	Product Size (bp)
***gyr*B**	Fw_TCCTCAACTCTGGCGTTTCT	59.99	237
	Rv_GGGGAATGTTGTTGGTGAAG	60.21	
***rpo*D**	Fw_GGTTATCTCCATCGCCAAGA	60.04	233
	Rv_TGAGCTTGTTGATGGTCTCG	59.98	
***rps*L**	Fw_CCACGGATCAGAACAACAGA	59.68	130
	Rv_CCCCGAAGAAGCCTAACTCT	59.84	
***opm*A**	Fw_TTGGTGCTGCTCACTTCAAC	60.03	229
	Rv_ATGGACAGATCCTTGGCAAC	59.93	
***rlm*L**	Fw_GAGCGCACTGGtGTCATCTA	60.02	265
	Rv_GCGCATTCTGAGAGAGATCC	60.07	

Target genes were selected based on the differential expression levels observed in the transcriptome sequencing data (Table 5):

- 5 genes over-expressed when exposed to TBT (5 μM and 50 μM TBT);
- 5 genes under-expressed when exposed to TBT (5 μM and 50 μM TBT);

- 3 genes under-expressed when exposed to 5 µM TBT and over-expressed when exposed to 50 µM TBT.
- 1 gene over-expressed at 5 µM TBT and under-expressed at 50 µM TBT.

The primers used to amplify the target genes are listed in Table 6.

Specific primers were designed based on the sequence of the genes to be analysed. These primers were designed using the OligoPerfect™ Designer (http://tools.invitrogen.com/content.cfm?pageid=9716) and the Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).

For assessment of the gene expression levels, qPCR was performed in the CFX96TM Real-Time System (Bio-Rad), coupled with the C1000TM Thermal Cycler (Bio-Rad). The SoFastTM EvaGreen® Supermix was used according to manufacturer's instructions. The components, respective quantities and the program used are shown in Table 7 and Table 8, respectively. The analysis included three biological and two technical replicates. A control sample (without the reverse transcriptase enzyme) and a non-template control (RNase-free water instead of cDNA) were included in the analysis.

Standard curves were obtained for each gene (target and reference) using serial dilutions of the cDNA samples. This allowed the assessment of the reaction efficiency.

The gene expression level was determined using relative quantification and calculated using ΔΔCt method (Biosystems 2004).

The qPCR data were then submitted to statistical analyses by one-way ANOVA (qbase^{PLUS}, Biogazelle).

Table 5 – Target genes selected for qPCR validation and respective expression ratios (nº reads in the presence of TBT/nº reads in the absence of TBT) for 5 and 50 µM of TBT). Green: over-expressed genes; red: under-expressed genes.

Interpro ID	Interpro description	Gene name	Ratio (5 µM)	Ratio (50 µM)
IPR000298	Cytochrome c oxidase, subunit III	B224_000468*	0.18	0.91
IPR000835	Transcription regulator HTH, MarR	AHA_0734**	0.0	7.5
IPR011032	GroES-like	*gro*S	12.33	36
IPR001327	Pyridine nucleotide-disulphide oxidoreductase, NAD-binding domain	AHA_2170**	4.0	124
IPR001623	Heat shock protein DnaJ, N-terminal	*dna*J	5.0	13
IPR002429	Cytochrome c oxidase subunit II C-terminal	B224_000472*	0.33	0.0
IPR002586	Cobyrinic acid a,c-diamide synthase	IYQ_23030*	0.14	0.22
IPR003423	Outer membrane efflux protein TolC	*tol*C	3.08	6.2
PR004323	Divalent ion tolerance protein, CutA1	*cut*A	8.5	0.0
IPR004360	Glyoxalase/bleomycin resistance protein/dioxygenase	ASA_1926**	0.095	2.5
IPR004670	Na+/H+ antiporter NhaA	*nha*A	0.19	0.0
IPR005123	Oxoglutarate/iron-dependent oxygenase	AHA_2405**	2.0	46
IPR007210	ABC-type glycine betaine transport system, substrate-binding domain	AHA_3374**	0.11	0.043
IPR007863	Peptidase M16, C-terminal	IYQ_07906*	0.0	34

***: ORF name**

****: ordered locus name**

Table 6 - Primers used to amplify each target gene, with respective melting temperature and product size.

Gene	Primer (5'-3')	Tm (ºC)	Product Size (bp)
B224_000468	Fw_TGATGGTCACCTTCTTGCTG	59.83	130
	Rv_GTACCGACCAGGGTGAAGAA	59.97	
AHA_0734	Fw_GAGGTGAGTGACGAGCTCAA	59.13	105
	Rv_ATAGAGTGCAGTCGGGGTGA	60.28	
***gro*S**	Fw_TCATCATTAAGCGCATCGAA	60.32	188
	Rv_GCCGTAACCTTCGTTGAAGA	60.25	
AHA_2170	Fw_AGTTTGTCAGCTTCGCCAAT	59.88	290
	Rv_GTGTAAACATGGGGGCTGTC	60.24	
***dna*J**	Fw_GCAAGACACTGCGTTTCTGA	60.18	231
	Rv_TCCAGCTCTTCGAGCAACTT	60.28	
B224_000472	Fw_CAcGAGCTCGATCCATACAA	59.82	100
	Rv_ATTCCGGATACACGAACAGC	59.96	
IYQ_23030	Fw_AAATGGCCAAGATGTCGTTC	59.94	256
	Rv_TTGTGATGGACGAAACGGTA	59.96	
***tol*C**	Fw_TGTCAGTCATGGTCCTGCTC	59.83	202
	Rv_TTGGCCAGGTAGTTGAGTCC	60.11	
***cut*A**	Fw_GACCTCATCAGCGAGCAACT	60.56	127
	Rv_TGATGATGAGCTGGATCTCG	59.90	
ASA_1926	Fw_AGCCAACACCATCAATCCTC	59.93	136
	Rv_GTGTTTGTGACGGGCTACCT	60.04	
***nha*A**	Fw_ACGATCTGGGCGTCATTATC	59.92	150
	Rv_ACCCGACCAACATGTAGAGG	59.84	
AHA_2405	Fw_GCCAGAAGGACATCAAGGAG	59.80	137
	Rv_GTACTGCTCCACCCAGGAGA	60.02	
AHA_3374	Fw_ACATAGCGCAGCTCAAGGAT	60.01	295
	Rv_ACTTCCAGCCAGACCACATC	60.12	
IYQ_07906	Fw_GGAAGACAAGGTCCATCTGC	59.66	224
	Rv_GGTTGGGATAGGCGTAGACA	59.96	

Table 7 – Components and respective volumes used in each qPCR reaction. The sequences of the primers used are shown in Table 4 and Table 6.

Component	Volume/Reaction (µL)
SsoFast EvaGreen supermix	10
Forward primer (500 nM)	1
Reverse primer (500 nM)	1
RNase-free water	7
cDNA template (samples)/RNase-free water (non-template control)	1

Table 8 - Program used in the qPCR.

	Temperature (ºC)	Time (seconds)	Nº Cycles
Enzyme activation	98	30	1
Denaturation	95	5	50
Annealing/extension	60	15	
Melting curve	65-95 (in 0.5ºC increments)	5/step	1

RESULTS AND DISCUSSION

10. Transcriptome analysis

10.1 Sequencing and data assembly

This procedure was performed to allow the identification of differentially expressed genes in the transcriptome of the Av27 strain following exposure to TBT (5 µM and 50 µM), since these genes may be involved in TBT resistance and degradation. The 454 Pyrosequencing assembly results are shown in Table 9. Figures 10-12 represent the distribution of reads for each condition analysed.

Table 9 - Pyrosequencing assembly results: number of reads, total number of bases and average read length.

	Control	**5 µM TBT**	**50 µM TBT**
Number of reads	120,260	72,800	92,710
Total number of bases	41,468,545	23,108,112	32,550,118
Average length (bp)	410.5	378.1	411.1

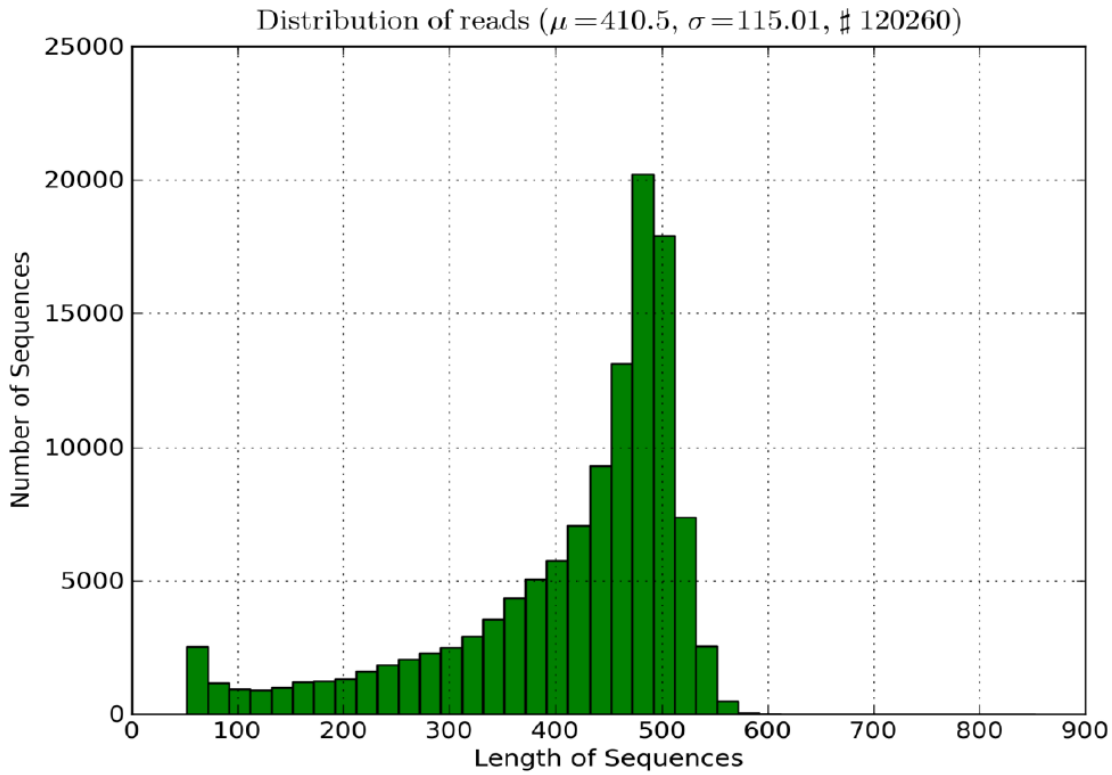


Figure 10 – Distribution of the length of the sequences resulting from pyrosequencing of *A. molluscorum* Av27's transcriptome in the control condition (non-exposed to TBT) (provided by Biocant).
µ: average length; σ: standard deviation; #: number of reads.

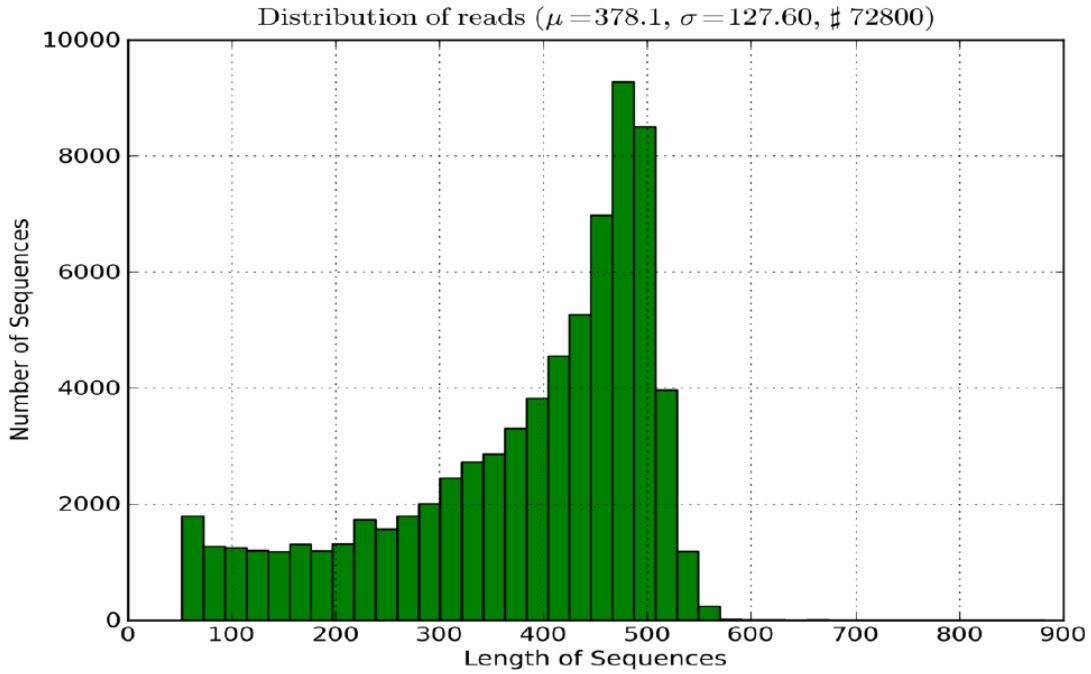


Figure 11 - Distribution of the length of the sequences resulting from pyrosequencing of *A. molluscorum* Av27's transcriptome exposed to 5 µM TBT (provided by Biocant).
µ: average length; σ: standard deviation; #: number of reads.

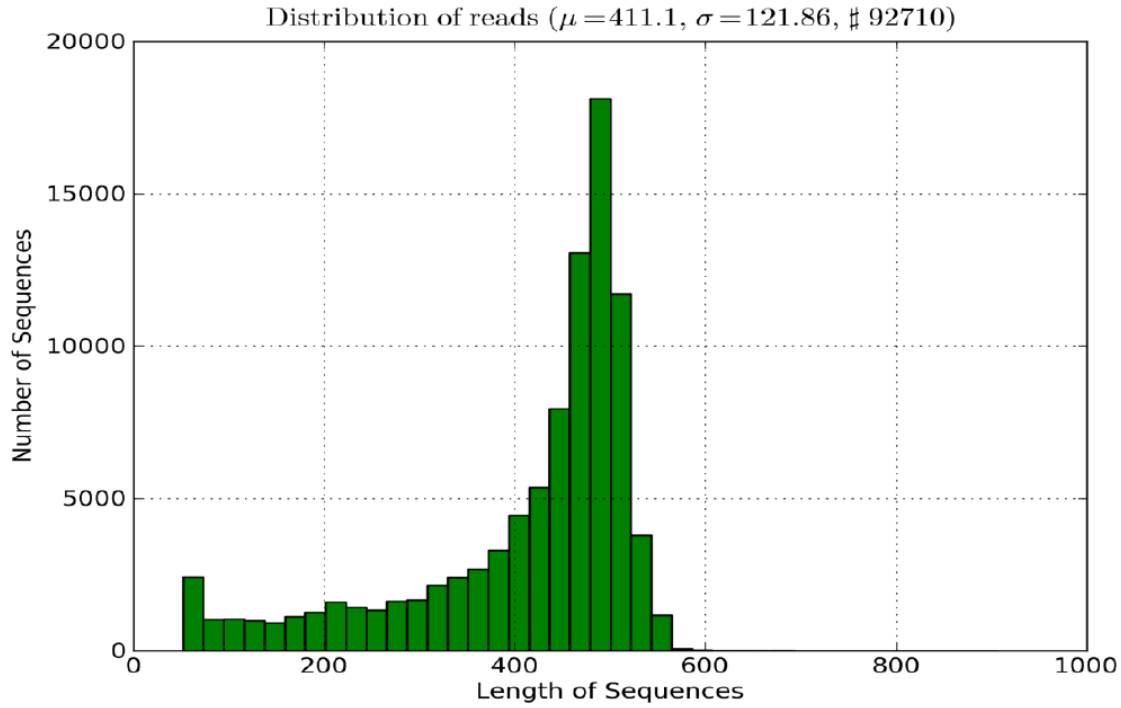


Figure 12 - Distribution of the length of the sequences resulting from pyrosequencing of *A. molluscorum* Av27's transcriptome exposed to 50 µM TBT (provided by Biocant).
µ: average length; σ: standard deviation; #: number of reads.

After sequencing, the reads were assembled into contigs. The results after assembly are shown in Table 10.

Table 10 – *A. molluscorum* Av27 transcriptome sequencing and assembly in the control sample and following TBT exposition (5 and 50 µM of TBT).

	Control	5 µM TBT	50 µM TBT
Number of reads	106,896	60,378	80,276
Total bases	34,656,300	18,312,500	26,942,400
Average read length after trimming (bp)	324	303	335
Number of contigs	1,360	1,147	1,325
Average contig length (bp)	1,056	878	982
Range of contig length (bp)	[77..16,243]	[141..13,769]	[133..13,973]
Number of contigs with >2 reads	1,360	1,147	1,325

All the contigs were then assigned to protein names and function using InterproScan (EMBL-EBI 2013), which also provided gene ontology identification. The annotation results are summarized in Table 11.

As previously shown, the transcriptome analysis provided the number of reads for each gene at each condition. Using this information, it was possible to calculate the expression ratio for the conditions under analysis (5 µM and 50 µM TBT). Some relevant genes and the respective expression ratios are presented in table A 1 (appendix). Expression ratio values ≥ 2 were considered to be over-expressed, while ratios < 0.5 were considered to be under-expressed. A p-value ≤ 0.05 was considered statistically significant.

Table 11 – *A. molluscorum* Av27 transcriptome annotation in the control sample and following TBT exposure (5 and 50 μM of TBT).

	Control	5 μM TBT	50 μM TBT
Number of contigs	1,360	1,147	1,325
Peptides with E-value < $1e^{-6}$ (a)	1,549	1,181	1,429
Remaining Peptides with frameDP (b)	336	226	306
Remaining Peptides with ESTscan (c)	143	148	169
Total number of Peptides (a+b+c)	2,028	1,555	1,904
Amino acid sequence assigned to GO terms	1,252	984	1,168
Amino acid sequence assigned InterPro terms	1,576	1,184	1,415
Amino acid sequence not assigned InterPro terms (from blastx E<$1e^{-6}$)	53	30	57
Amino acid sequence not assigned InterPro terms (from FrameDP)	259	199	273
Amino acid sequence not assigned InterPro terms (from ESTScan)	140	142	159

11. qPCR validation

11.1 RNA concentration

The RNA concentration and purity are presented in Table 12.

Table 12 - Concentration and purity of A. molluscorum Av27's RNA in the control sample (non-exposed) and following exposure to TBT (5 and 50 µM TBT). $A_{260}/A_{280} \geq 2$: pure RNA; $A_{260}/A_{280} < 2$: contaminated RNA.

Sample	Biological Replicate	Concentration (ng/µL)	A_{260}/A_{280}
Control	1	1550.1	1.99
	2	1199.2	1.96
	3	1020.7	2.03
5 µM TBT	1	1098.5	2.05
	2	1379.4	2.06
	3	1167.0	2.04
50 µM TBT	1	1104.5	2.09
	2	1072.8	2.08
	3	778.1	2.08

11.2 Standard Curves

Standard curves allow to assess the efficiency of the PCR reaction. In fact, efficiency is calculated by the slope of the standard curve:

$$Efficiency = 10^{\left(-\frac{1}{slope}\right)} - 1$$

Ideally, the efficiency should be 100% (slope = -3.32), but this value is difficult to achieve. Hence, efficiency values between 90 and 110% are acceptable.

The correlation coefficient of the curve should be ≈ 1 and the y-intercept value provides information about the theoretical limit of detection of the reaction (C_T correspondent to the minimum copy number that gives rise to statistically significant amplification) (Invitrogen 2008).

Efficiency, correlation coefficient, slope and y-intercept values are presented in Table 13 and Table 14 for the reference genes and the target genes, respectively.

Table 13 - Efficiency, correlation coefficient, slope and y-intercept values obtained from the standard curve of the reference genes.

Genes	Efficiency	r^2	slope	y-int
***gyr*B**	99.7%	0.991	-3.330	23.315
***rpo*D**	103.8%	0.951	-3.233	30.429
***rps*L**	97.3%	0.996	-3.389	23.324
***opm*A**	96.0%	0.993	-3.421	23.841
***rlm*L**	97.6%	0.993	-3.382	24.129

Table 14 - Efficiency, correlation coefficient, slope and y-intercept values obtained from the standard curve of the target genes.

Genes	Efficiency	r^2	slope	y-int
B224_000468	100.6%	0.993	-3.308	23.138
AHA_0734	98.3%	0.998	-3.363	23.817
***gro*S**	104.9%	0.995	-3.210	22.488
AHA_2170	97.2%	0.957	-3.392	33.843
***dna*J**	110.0%	0.997	-3.103	21.843
B224_000472	97.6%	0.985	-3.382	24.995
IYQ_23030	99.7%	0.988	-3.329	22.059
***tol*C**	97.5%	0.998	-3.383	23.354
***cut*A**	98.1%	0.994	-3.369	22.034
ASA_1926	96.4%	0.985	-3.411	23.538
***nha*A**	105.8%	0.993	-3.191	24.964
AHA_2405	100.14%	0.998	-3.312	22.819
AHA_3374	98.5%	0.993	-3.359	27.247
IYQ_07906	99.8%	0.995	-3.327	21.191

Based on the reaction efficiency and correlation coefficient, only B224_000468, AHA_0734, *gro*S, B224_000472, IYQ_23030, tolC, *cut*A, ASA_1926, *nha*A, AHA_2405, AHA_3374 and IYQ_07906 were selected for qPCR validation.

11.3 Reference genes – Stability study

For relative quantification, the use of reference genes to normalize the expression levels between experiments is required. The expression levels of the reference genes should remain constant at all points of the experiment and at all the conditions tested (Invitrogen 2008).

The stability of the reference genes used in this study was assessed using geNorm (qbase PLUS, Biogazelle) and the results are presented in Table 15. Ideally, the M value should be <0.5 and the coefficient of variation (CV) <0.2, since these values indicate low variation among the conditions tested.

Table 15 - M value and coefficient of variation (CV) for each reference gene.

	***gyr*B**	***rpo*D**	***rps*L**	***opm*A**	***rlm*L**
M value	0.267	0.338	0.402	0.308	0.232
CV	0.033	0.140	0.198	0.127	0.080

The analysis performed by geNorm indicated that the use of two reference genes is ideal. The genes *gyr*B and *rlm*L were recommended, so these genes were selected as the reference genes for the qPCR analysis.

11.4 qPCR results

In qPCR, relative quantification doesn't provide the exact starting quantity of the target gene, but allows comparison between gene expression in a treated sample and an untreated one. Hence, the expression ratios between the samples treated with TBT (5 µM and 50 µM TBT) and the control sample (no TBT) were calculated using statistical tools (One-way ANOVA). Expression ratio values ≥1 were considered to be over-expressed, while ratios <1 were considered to be under-expressed. A p-value ≤0.05 was considered statistically significant.

In this analysis, the relative expression of six genes revealed the same tendency as the relative expression of the transcriptome, thus validating its results. Five genes validated only one of the two conditions tested and one didn't validate the results provided by the transcriptome analysis (Table 16). Since the qPCR experiment validated the gene expression levels revealed by the transcriptome results, these can be used to infer about possible genes that might be involved in TBT resistance and/or degradation.

Table 16 – Comparison between qPCR validation results and transcriptome analysis: p-value and expression ratios obtained for each gene

Green: over-expressed genes (expression ratio ≥2); red: under-expressed genes (expression ratio <0.5).

V: qPCR validated transcriptome results; NV: qPCR did not validate transcriptome analysis.

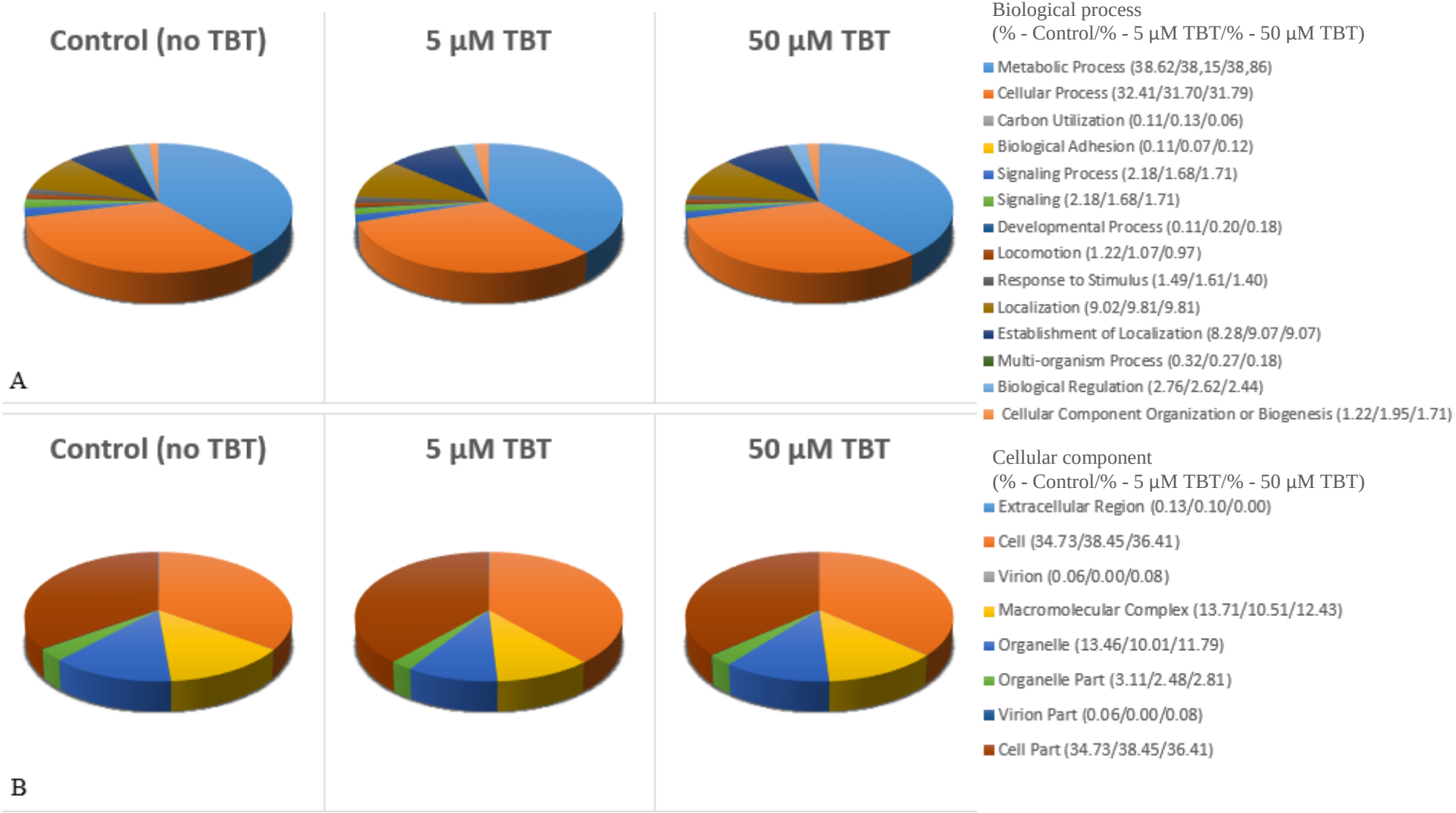
	qPCR (One-way ANOVA)			Transcriptome analysis			Validation result	
Gene	**p-value**	**Ratio 5 µM**	**Ratio 50 µM**	**p-value**	**Ratio 5 µM**	**Ratio 50 µM**	**5 µM**	**50 µM**
B224_000468	0.213	0.813	0.607	9.115E-03	0.182	0.909	V	V
AHA_0734	0.273	0.173	1.845	6.493E-11	0.000	7.500	V	V
***gro*S**	0.163	0.875	1.307	1.112E-23	12.33	36.00	NV	V
B224_000472	0.575	1.036	0.905	1.334E-06	0.279	0.000	NV	V
IYQ_23030	0.088	0.234	0.374	2.828E-15	0.144	0.225	V	V
***tol*C**	0.606	1.045	2.454	1.208E-20	3.080	6.200	V	V
***cut*A**	0.223	1.136	1.737	2.853E-03	8.500	0.000	V	NV
ASA_1926	3.81E-03	2.293	1.111	0.000E-00	0.095	2.500	NV	V
***nha*A**	0.074	1.436	1.712	1.203E-12	0.190	0.000	NV	NV
AHA_2405	0.192	1.004	3.020	5.580E-19	2.000	46.00	V	V
AHA_3374	0.399	0.473	0.705	0.000E+00	0.115	0.043	V	V
IYQ_07906	0.120	1.061	1.442	1.150E-14	0.000	35.00	NV	V

12. Differential gene expression analysis

12.1 Functional analysis

The transcriptome of *A. molluscorum* Av27 following exposure to TBT (5 µM and 50 µM) was studied in order to identify the genes involved in TBT resistance and degradation. The GO annotations (e-value$\leq 10^{-6}$) provided a valuable resource for the investigation of specific processes, functions or cellular structures involved in the resistance and degradation of TBT in the Av27 strain.

The biological process, cellular component and molecular function for each condition tested (control, 5 µM and 50 µM TBT exposure) were analyzed (Figure 13). However, the similarity between the results obtained for each condition makes it difficult to draw any conclusions. Hence, the number of over and under-expressed genes was analyzed in order to better understand the effect of TBT on the different functional categories defined (Figure 14). The analysis of Figure 14 shows that, at 5 µM TBT, the number of repressed genes is much higher than the number of induced genes, which is probably related to an energy saving and/or survival mechanism by the cell. However, at 50 µM TBT the cell faces the need to activate mechanisms of resistance and/or degradation to survive in these conditions. Consequently, more genes are found over-expressed at 50 µM TBT than at 5 µM TBT. At both TBT concentrations, the functional category showing higher variation in both over and under-expressed genes was that related with enzymatic activity, followed by transport, binding and oxidation-reduction. These categories included genes that are mainly over-expressed at 50 µM, suggesting their possible involvement in TBT resistance and degradation. In fact, it has been suggested that TBT can be exported from the cell through efflux pumps (Jude, Arpin et al. 2004) and that it is degraded through dealkylation and demethylation processes (Barug 1981), which involves enzymatic machinery possibly encoded by genes belonging to these functional categories.


Control (no TBT)
5 µM TBT
50 µM TBT
A
Control (no TBT)
5 µM TBT
50 µM TBT
B
Biological process
(% - Control/% - 5 µM TBT/% - 50 µM TBT)
Metabolic Process (38.62/38,15/38,86)
Cellular Process (32.41/31.70/31.79)
Carbon Utilization (0.11/0.13/0.06)
Biological Adhesion (0.11/0.07/0.12)
Signaling Process (2.18/1.68/1.71)
Signaling (2.18/1.68/1.71)
Developmental Process (0.11/0.20/0.18)
Locomotion (1.22/1.07/0.97)
Response to Stimulus (1.49/1.61/1.40)
Localization (9.02/9.81/9.81)
Establishment of Localization (8.28/9.07/9.07)
Multi-organism Process (0.32/0.27/0.18)
Biological Regulation (2.76/2.62/2.44)
Cellular Component Organization or Biogenesis (1.22/1.95/1.71)
Cellular component
(% - Control/% - 5 µM TBT/% - 50 µM TBT)
Extracellular Region (0.13/0.10/0.00)
Cell (34.73/38.45/36.41)
Virion (0.06/0.00/0.08)
Macromolecular Complex (13.71/10.51/12.43)
Organelle (13.46/10.01/11.79)
Organelle Part (3.11/2.48/2.81)
Virion Part (0.06/0.00/0.08)
Cell Part (34.73/38.45/36.41)

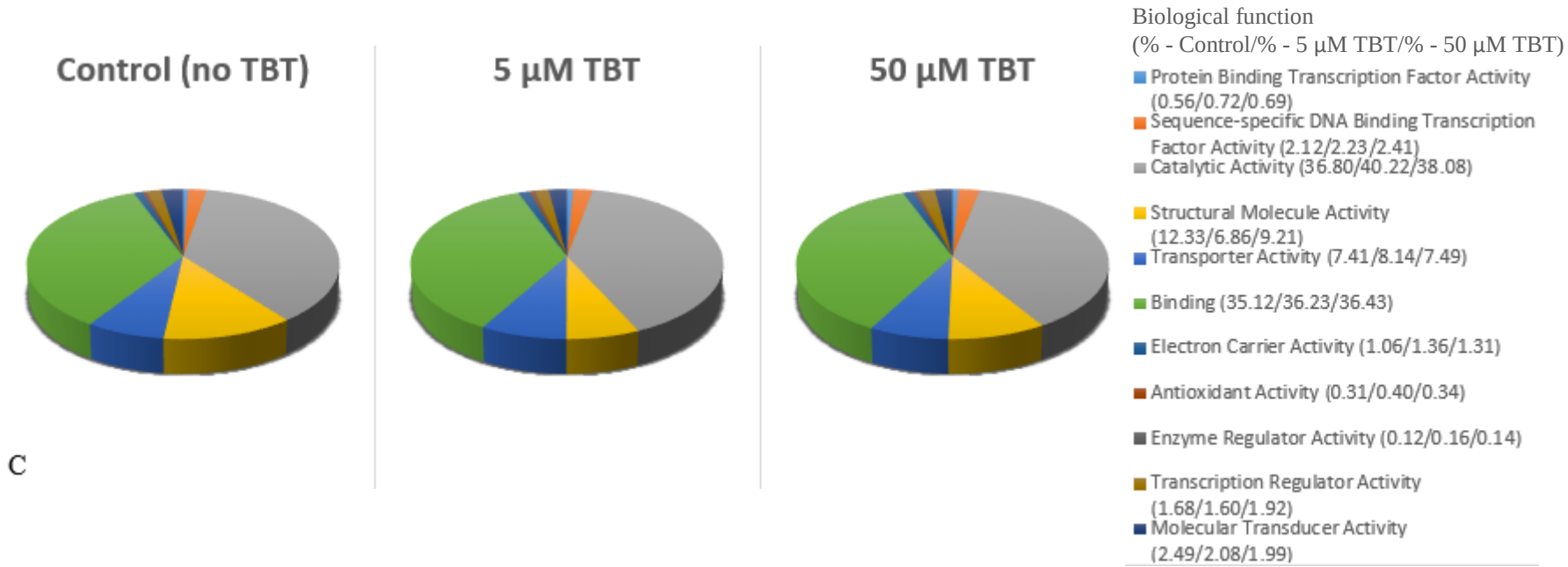


Figure 13 – Gene ontology (GO). Percentage of gene ontology annotations for *A. molluscorum* Av27 sequences: biological process (A), cellular component (B) and molecular function (C).

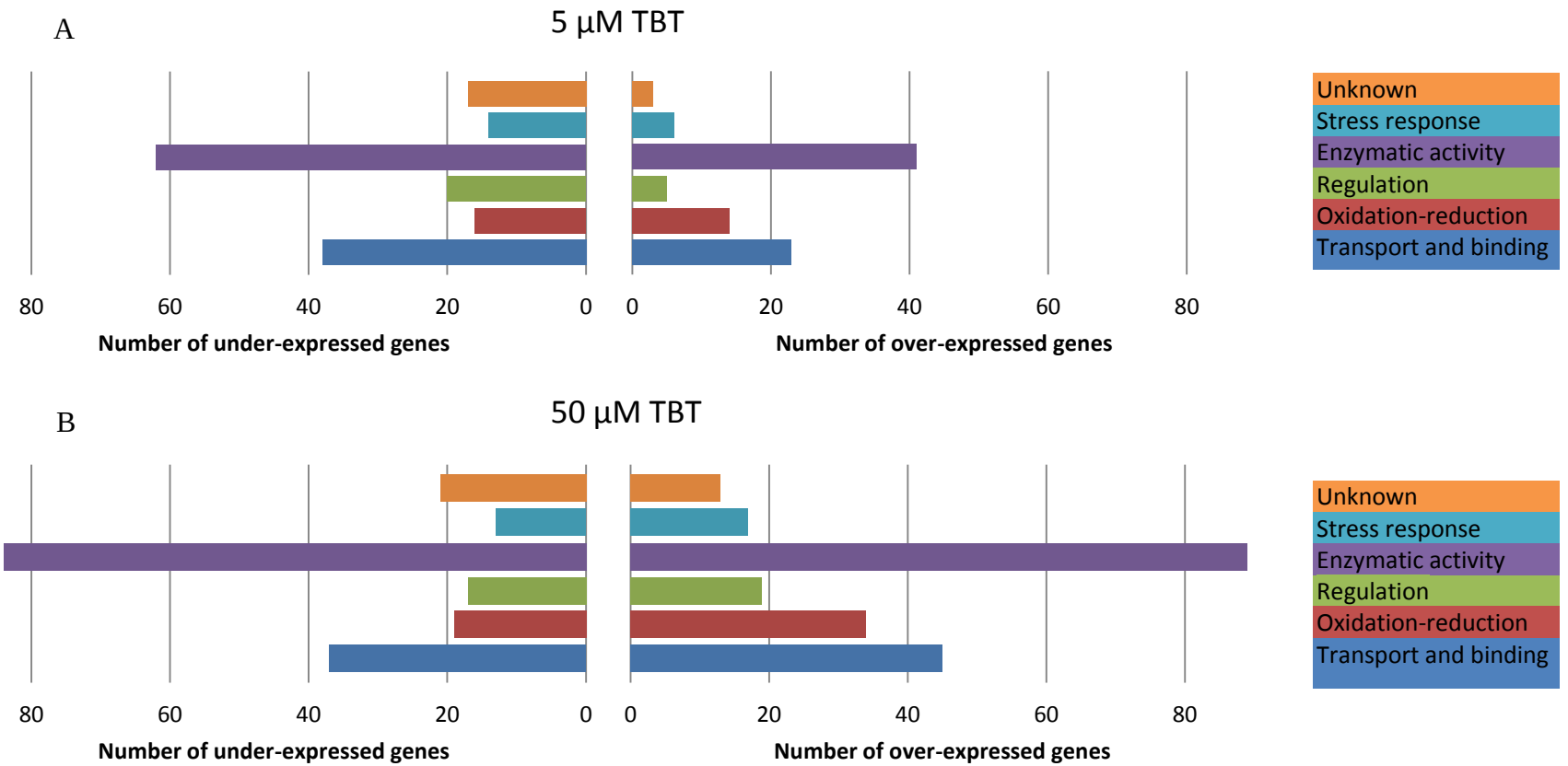


Figure 14 - Functional categories of over and under-expressed genes following exposure of *A. molluscorum* Av27 to 5 µM (A) and 50 µM of TBT (B).

12.2 Comparison with previous studies

12.2.1 Expression levels of SugE in *Aeromonas molluscorum* Av27

Cruz et al (2013) identified a gene in *Aeromonas molluscorum* Av27 which was over-expressed in the presence of 500 μM of TBT when cells reached the early growth phase (OD_{600nm} = 0.2). This gene presented homology (84 %) with *sug*E gene from *Aeromonas hydrophila*, encoding SugE protein. SugE protein belongs to the small multidrug resistance (SMR) family (IPR000390), which is located in the inner membrane and is involved in the efflux of lipophilic compounds (Sikora and Turner 2005).

Considering the transcriptome data, the SMR family (IPR000390) is under-expressed in this study. This result is in accordance with that obtained by Cruz (2013), which described that *sug*E is not significantly over-expressed when cells are grown to the mid log phase (OD_{600nm} = 0.5) in any of the TBT concentrations tested. Cruz suggested that Av27-*sug*E is only over-expressed in the early growth phase due to its involvement in a rapid response to TBT. In the present study, the Av27 strain was grown to the mid log phase, and probably other genes are involved in TBT resistance during this growth phase.

12.2.2 Comparison of TBT resistance in *Pseudomonas aeruginosa* 25W and *A. molluscorum* Av27

The TBT-regulated genes in *Pseudomonas aeruginosa* 25W, a TBT-resistant strain, were studied by Dubey, Tokashiki et al. (2006). The authors used DNA microarrays to analyse the gene expression profile upon exposure to TBT.

Fukushima and co-workers also studied the effect of TBT (50 μM and 500 μM) in *P. aeruginosa* 25W cells that were grown to mid-log phase, and identified some up-regulated and down-regulated genes following TBT-exposure. Some of these differentially expressed genes were selected to perform quantitative analyses (qPCR) (Fukushima, Dubey et al. 2009).

The results obtained from the pyrosequencing of the transcriptome of *A. molluscorum* Av27 were compared to those obtained by Dubey in the microarrays study and are shown in Table 17.

Table 17 - Comparison of the results obtained through the analysis of the transcriptome of *A. molluscorum* Av27 and the transcriptome of *P. aeruginosa* 25W, focusing on the genes that showed altered expression levels upon TBT exposure. The genes that presented different relative expression are highlighted in red.

Microarrays study of *P. aeruginosa* 25W			**Pyrosequencing study of *A. molluscorum* Av27**			
PA number	**Description**	**Relative Expression**	**InterPro ID**	**Description**	**Relative Expression**	**p-value**
PA5348	Probable DNA-binding protein	Over-expressed (500 µM TBT)	IPR000119	Histone-like bacterial DNA-binding protein	Over-expressed (50 µM TBT)	>0.05
PA5178	Conserved hypothetical protein	Over-expressed (500 µM TBT)	IPR002482	Peptidoglycan-binding Lysin subgroup	Over-expressed (5 µM and 50 µM TBT)	1.53444E-14
PA1983	Cytochrome c550	Over-expressed (500 µM TBT)	IPR009056	Cytochrome c domain	Over-expressed (50 µM TBT)	6.56960E-06
PA0329	Conserved hypothetical protein	Over-expressed (500 µM TBT)	IPR010879	Domain of unknown function DUF1508	Over-expressed (5 µM and 50 µM TBT)	>0.05
PA3600	Ribosomal protein L36	Under-expressed (500 µM TBT)	IPR000473	Ribosomal protein L36	Under-expressed (50 µM TBT)	>0.05
PA4242	50S ribosomal protein L36					
PA2966	Acyl carrier protein	Under-expressed (500 µM TBT)	IPR009081	Acyl carrier protein-like	Under-expressed (5 µM and 50 µM TBT)	>0.05

PA3601	50S ribosomal protein L31 type B	Under-expressed (500 µM TBT)	IPR002150	Ribosomal protein L31	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA1159	Probable cold-shock protein	Under-expressed (500 µM TBT)	IPR002059	Cold-shock protein, DNA-binding	Under-expressed (5 µM and 50 µM TBT)	1.31077E-07
PA3450 PA0139	Probable antioxidant protein Alkyl hydroperoxide reductase subunit C	Under-expressed (500 µM TBT)	IPR000866	Alkyl hydroperoxide reductase subunit C/ Thiol specific antioxidant	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA1053	Outer membrane lipoprotein	Under-expressed (500 µM TBT)	IPR004658	Outer membrane lipoprotein Slp	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA1830	Putative sterol carrier protein	Under-expressed (500 µM TBT)	IPR003033	SCP2 sterol-binding domain	Under-expressed (5 µM TBT)	>0.05
PA4385	GroEL protein	Under-expressed (500 µM TBT)	IPR001844	Chaperonin Cpn60	Over-expressed (50 µM TBT)	1.83579E-22
PA1804	DNA-binding protein HU	Under-expressed (500 µM TBT)	IPR000119	Histone-like bacterial DNA-binding protein	Under-expressed (5 µM and 50 µM TBT)	>0.05

PA4265 PA4277	Elongation factor TU	Under-expressed (500 µM TBT)	IPR004161	Translation elongation factor EFTu/EF1A, domain 2	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA4611	Hypothetical protein	Under-expressed (50 µM and 500 µM TBT)	IPR007420	Protein of unknown function DUF465	Under-expressed (5 µM TBT)	1.69502E-02
PA4944	Conserved hypothetical protein	Under-expressed (500 µM TBT)	IPR010920	Like-Sm ribonucleoprotein (LSM)-related domain	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA4386	GroES protein	Under-expressed (500 µM TBT)	IPR011032	GroES-like	Over-expressed (5 µM and 50 µM TBT)	1.71186E-03
PA1557	Probable cytochrome oxidase subunit (cbb3-type)	Under-expressed (500 µM TBT)	IPR000883	Cytochrome c oxidase, subunit I	Under-expressed (5 µM TBT)	>0.05
PA2853	Outer membrane lipoprotein OprI precursor	Under-expressed (500 µM TBT)	IPR004658	Outer membrane lipoprotein Slp	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA0073	Probable ATP-binding component of ABC transporter	Under-expressed (500 µM TBT)	IPR003439	ABC transporter-like	Under-expressed (5 µM and 50 µM TBT)	>0.05

PA4795	Putative protein	Under-expressed (500 µM TBT)	IPR003787	Sulphur relay, DsrE/F-like protein	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA4272	50S ribosomal protein L10	Under-expressed (500 µM TBT)	IPR001790	Ribosomal protein L10/acidic P0	Under-expressed (5 µM and 50 µM TBT)	1.70626E-06
PA0456	Probable cold-shock protein	Under-expressed (500 µM TBT)	IPR002059	Cold-shock protein, DNA-binding	Under-expressed (5 µM and 50 µM TBT)	1.31077E-07
PA4264	30S ribosomal protein S10	Under-expressed (500 µM TBT)	IPR001848	Ribosomal protein S10	Under-expressed (50 µM TBT)	1.69024E-09
PA3202	Protein yciI	Under-expressed (500 µM TBT)	IPR011008	Dimeric alpha-beta barrel	Under-expressed (5 µM TBT)	>0.05
PA3742	50S ribosomal protein L19	Under-expressed (500 µM TBT)	IPR008991	Translation protein SH3-like	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA5288	Nitrogen regulatory protein P-II 2	Under-expressed (500 µM TBT)	IPR011322	Nitrogen regulatory PII-like, alpha/beta	Under-expressed (50 µM TBT)	1.19775E-02
PA3309	Universal stress protein UspA and related nucleotide-binding proteins	Under-expressed (500 µM TBT)	IPR006016	UspA	Under-expressed (5 µM and 50 µM TBT)	1.10400E-10

PA3397	Ferredoxin-NADP+ reductase	Under-expressed (500 µM TBT)	IPR001433	Oxidoreductase FAD/NAD(P)-binding	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA4176	Peptidyl-prolyl cis-trans isomerase C2	Under-expressed (500 µM TBT)	IPR000297	Peptidyl-prolyl cis-trans isomerase, PpiC-type	Over-expressed (5 µM and 50 µM TBT)	1.71815E-12
PA4257	30S ribosomal protein S3	Under-expressed (500 µM TBT)	IPR004088	K Homology, type 1	Under-expressed (5 µM and 50 µM TBT)	5.05344E-25
PA0357	Formamidopyrimidine-DNA glycosylase	Under-expressed (500 µM TBT)	IPR000214	Zinc finger, DNA glycosylase/AP lyase-type	Under-expressed (50 µM TBT)	>0.05
PA4322	MoxR-like ATPases	Under-expressed (500 µM TBT)	IPR011703	ATPase, AAA-3	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA4255	50S ribosomal protein L29	Under-expressed (500 µM TBT)	IPR001854	Ribosomal protein L29	Under-expressed (5 µM and 50 µM TBT)	>0.05
PA0779	Probable ATP-dependent protease	Under-expressed (500 µM TBT)	IPR008269	Peptidase S16, Lon C-terminal	Under-expressed (5 µM and 50 µM TBT)	>0.05

PA0715	Reverse transcriptase	Under-expressed (500 µM TBT)	IPR000123	RNA-directed DNA polymerase (reverse transcriptase), msDNA	Under-expressed (5 µM and 50 µM TBT)	1.09310E-03
PA2227	Probable transcriptional regulator	Under-expressed (500 µM TBT)	IPR009057	Homeodomain-like	Under-expressed (5 µM and 50 µM TBT)	1.70532E-02
PA3150	LPS biosynthesis protein WbpG	Under-expressed (500 µM TBT)	IPR014729	Rossmann-like alpha/beta/alpha sandwich fold	Under-expressed (5 µM and 50 µM TBT)	1.10400E-10
PA2228	6-aminohexanoate-dimer hydrolase	Under-expressed (500 µM TBT)	IPR012338	Beta-lactamase/transpeptidase-like	Under-expressed (5 µM and 50 µM TBT)	>0.05

The comparison analysis revealed that, in a total of 81 genes, 39 of the genes present in the microarrays study did not have a correspondence with genes that were found up in the transcriptome of *A. molluscorum* Av27 (data not shown), where 33 of these unmatched genes correspond to hypothetical proteins. Among the remaining 42 genes, only those encoding peptityl-prolyl cis-trans isomerase C2, GroES protein and GroEL protein showed a differential expression level in both studies: these genes were under-expressed in *P. aeruginosa* 25W and over-expressed in the Av27 strain.

The analysis of the microarrays results in *P. aeruginosa* 25W show the down-regulation of several transcription-related genes, suggesting that high TBT concentrations generate stresses that result in an inhibition of transcription of these genes (Dubey, Tokashiki et al. 2006). As shown, *A. molluscorum* Av27 also presents several transcription-related genes that are under-expressed upon exposure to TBT. Thus, as is the case in *P. aeruginosa* 25W, TBT also seems to affect the transcription of some genes in *A. molluscorum* Av27.

12.2.3 *Aeromonas hydrophila* ATCC7966 and *A. molluscorum* Av27; do they share the same mechanism of resistance to TBT?

Hernould et al (2008) described the presence of the AheABC efflux pump in *Aeromonas hydrophila* that is associated with the intrinsic resistance of *A. hydrophila* ATCC7966^T to several compounds. This tripartite efflux pump belongs to the resistance-nodulation-cell division (RND) family. The substrate specificity of the efflux pump was assessed, and TBT was one of the compounds to be analysed.

In Hernould's study, it was shown that AheABC is encoded by *aheA, aheB* and *aheC* genes, which present the same orientation and are organized in an operon-like structure. Besides, the authors demonstrated that AheABC has the ability to export TBT from the cell. It is noteworthy to mention that AheABC is able to export TBT, but with much lower efficiency than the homologous AcrB system in *E. coli*. In fact, Hernould also suggests the presence of other efflux pumps involved in the intrinsic drug resistance in *A. hydrophila* ATCC7966^T (Hernould, Gagné et al. 2008).

Considering the role of AheABC in *A. hydrophila* ATCC7966^T, it is pertinent to think that the AheABC efflux pump may be involved in the TBT resistance in *A. molluscorum* Av27. Acriflavin resistance protein (IPR001036), which is homologue to

AheABC, is found in *A. molluscorum* Av27. However, more than one contig presented homology with this protein, and the different contigs showed different expression levels. In fact, some contigs were found under-expressed when Av27 strain was exposed to both TBT concentrations, while others were found to be over-expressed following exposure to 50 μM of TBT. Hence, the acriflavin resistance protein may be involved in TBT resistance in Av27 strain. However, and as suggested by various authors, several mechanisms of TBT resistance seem to be presented in different bacterial species, thus it can be assumed that in *A. molluscorum* Av27 other genes should also contribute to TBT resistance.

12.3 Other proteins potentially involved in TBT resistance/degradation in *Aeromonas molluscorum* Av27

12.3.1 Resistance mechanisms: relationship between TBT and stress conditions

Heat-shock proteins (Hsp), also known as molecular chaperones, have the role of maintaining proper protein folding within the cell and to re-fold denaturated proteins. Hsps are also involved in the prevention of protein aggregation, degradation and trafficking and in the maintenance of signalling proteins in their correct conformation. Although these proteins were first related to high temperatures exposition, heat-shock proteins are in fact induced by a variety of cellular stresses (EMBL-EBI 2013).

Some heat-shock proteins were identified in the transcriptome of *A. molluscorum* Av27, namely Hsp70 (IPR001023), Hsp90 (IPR001404) and Hsp20 (IPR002068). The presence of these proteins may explain the fact that this bacterium is able to grow in a wide range of temperatures (4 to 37ºC) (Cruz 2012). Furthermore, in the present study, these hsps were over-expressed when *A. molluscorum* Av27 was exposed to 50 μM TBT. Since hsps are involved in stress response and TBT is a stress agent, these results suggest that hsps may also play a role in TBT resistance in Av27 strain. In fact, as with other types of stress, hsps probably act by re-folding denaturated proteins and preventing protein aggregation (EMBL-EBI 2013), thus correcting the damage caused by TBT.

The analysis of the transcriptome of *A. molluscorum* Av27 revealed the presence of other hsps that were found to be over-expressed when cells are exposed to TBT as, for

instance, heat-shock protein HslU (IPR004491) and heat-shock protein DnaJ (IPR001623). HslU eliminates misfolded or damaged proteins and controls the levels of some regulatory proteins. DnaJ stimulates Hsp70 (EMBL-EBI 2013). HslU was over-expressed when *A. molluscorum* Av27 was exposed to 50 µM TBT, and DnaJ was over-expressed upon exposure to both TBT concentrations (5 µM and 50 µM). These results also suggest that heat-shock proteins may play a role in *A. molluscorum* Av27's resistance to TBT.

12.3.2 Resistance mechanisms: interplay between TBT and heavy metals

Aeromonas molluscorum Av27 is known to be resistant to some heavy metals, namely mercury, copper, zinc and cadmium (Cruz, Caetano et al. 2007). In fact, the analysis of the bacterium's transcriptome revealed the presence of a heavy metal-associated domain, HMA (IPR006121). This domain is found in heavy metal transport and detoxification proteins. Some of these proteins are actually involved in bacterial resistance to toxic heavy metals, such as lead and cadmium (EMBL-EBI 2013).

Other proteins involved in heavy metals resistance are CutA1 (IPR004323), which is thought to be involved in cellular tolerance to copper; ABC transporters (IPR001140), which present the ability to export ions from the cell; CorA and ZntB transporters (IPR002523), which mediate the transport of magnesium and zinc, respectively; and cation efflux proteins (IPR002524), which increase tolerance to divalent metal ions (EMBL-EBI 2013). These proteins can be found in the transcriptome of *Aeromonas molluscorum* Av27, thus confirming its involvement in heavy metal resistance, as described by Cruz et al. (2007).

The expression levels of HMA, CutA1 and cation efflux proteins were not affected by the presence of TBT. However, the ABC transporters and the CorA and ZntB proteins presented higher expression levels when the bacterium was exposed to TBT (5 µM or 50 µM TBT). Hence, it can be suggested that the TBT resistance on Av27 strain is probably associated with metal resistance, as previously suggested (Suzuki, Fukagawa et al. 1992).

12.3.3 Resistance mechanisms: relationship between TBT and antibiotics

As mentioned in section 4, *A. molluscorum* Av27 is resistant to some antibiotics, such as penicillin, amoxicillin/clavulanic acid and cephalothin (Cruz 2012). These results are confirmed by the presence of a multiple antibiotic resistance protein, MarC (IPR002771) in the transcriptome of *A. molluscorum* Av27. The MarC is an integral to membrane protein and it is thought to be a transporter, conferring resistance to antibiotics (EMBL-EBI 2013). This protein is involved in antibiotic resistance mechanism in Av27 strain, but its expression does not seem to be affected by TBT.

Jude et al. (2004) and Hernould et al. (2008) associated TBT and antibiotic resistance, since some efflux pumps involved in TBT resistance are also able to extrude antibiotics. The analysis of the transcriptome of *A. molluscorum* Av27 revealed the presence of an outer membrane efflux protein (IPR003423) that exports several substrates in Gram negative bacteria (EMBL-EBI 2013). This efflux protein was over-expressed when the cells were exposed to TBT, suggesting that TBT is probably one of the substrates exported by this protein. Likewise, the acriflavin resistance protein (IPR001036) also found in the Av27's transcriptome has higher expression levels when exposed to 50 μM TBT. This protein is part of a multi-drug efflux system that possibly protects the cells against hydrophobic inhibitors (such as TBT) and it also exports some antibiotics (EMBL-EBI 2013). These results confirm that efflux pumps can be simultaneously involved in TBT and in antibiotic resistance.

12.3.4 TBT degradation – proposed mechanism

A. molluscorum Av27 has the ability to degrade TBT into less toxic compounds, namely DBT and MBT (Cruz, Caetano et al. 2007). It is possible that this degradation involves dealkylation and demethylation, similar to what it is observed in other TBT-degrading organisms (Barug 1981).

The TBT degradation process might involve the enzymatic cleavage of the Sn-C bond. However, no enzyme catalyzing this cleavage reaction has been described so far. Nevertheless, it has already been shown that butane is a product of DBT degradation, which suggests that the process occurs by dealkylation rather than by demethylation (Inoue,

Takimura et al. 2003). No genes related to these processes were found in the transcriptome of Av27 strain.

Based on what is described in the literature, Cruz (2007) proposed that TBT is degraded into DBT and MBT in siderophore-like structures. In fact, it has been shown that triphenyltin can be degraded by pyoverdine, a peptide siderophore that presents the ability to chelate and transport iron (Meyer 2000; Inoue, Takimura et al. 2003). When the transcriptome of Av27 strain was screened for the presence of proteins similar to pyoverdine, no results were retrieved. However, there are many proteins of unknown function in the transcriptome of *A. molluscorum* Av27 that are significantly over-expressed following exposure to TBT (IPR007497, IPR009809, IPR016596, IPR002549, IPR005358, among others). One of these non-annotated proteins may be involved in TBT degradation.

Following degradation, DBT and MBT may be extruded from the cell and released to the culture media (Cruz, Caetano et al. 2007). It is possible that this process is mediated by some efflux pumps and transporter proteins. SugE, an inner membrane protein, is of particular interest, since it is known to be involved in the TBT resistance mechanism (Cruz, Micaelo et al. 2013). Thus, this protein may be responsible for the transport of DBT and MBT (lipophilic compounds) to the periplasmic space. Then, the outer membrane efflux protein TolC may be involved in the efflux of these degradation compounds from the cell. This protein was shown to be over-expressed in Av27 strain following exposure to TBT (Table 16), suggesting a possible role in the TBT resistance/degradation mechanism.

In conclusion, the TBT degradation mechanism probably involves the capture of this compound into siderophore-like structures, where the break of the Sn-C takes place. This degradation process leads to the formation of two types of products:

- DBT and MBT, which are extruded from the cell (Cruz, Caetano et al. 2007);
- butane, which is probably degraded and used as carbon source (Cruz, Caetano et al. 2007).

12.4 Genes with potential to be used in relevant applications

12.4.1 Development of bioreporters

The analysis of the transcriptome of *A. molluscorum* Av27 allowed the identification of some genes with significantly high expression levels when the cells were exposed to TBT and when compared with cells not exposed. Thus, these genes or their respective promoters have potential to be used as sensor elements for the construction of bioreporters to detect TBT in the environment (Table 18).

Table 18 – Proteins encoded by genes potentially useful for the construction of bioreporters.

InterPro ID	Description	Ratio (5 µM TBT)	Ratio (50 µM TBT)	Gene Ontology
IPR005123	Oxoglutarate/iron-dependent oxygenase	2	46	Oxidation-reduction
IPR007329	FMN-binding	2	15	FMN binding
IPR005480	Carbamoyl-phosphate synthetase, large subunit, oligomerisation	3	8	Catalytic activity
IPR004565	Outer membrane lipoprotein LolB	0	9	Protein transport
IPR012394	Aldehyde dehydrogenase NAD(P)-dependent	0	8	Oxidation-reduction
IPR007267	GtrA-like protein	0	12	Polysaccharide biosynthetic process; transport
IPR006660	Arsenate reductase-like	0	10	Transport; response to stress
IPR013116	Acetohydroxy acid isomeroreductase, catalytic	1	9	Oxidation-reduction
IPR019545	DM13 domain	0	18	Oxidation-reduction

Among the selected genes, the one that encodes for oxoglutarate/iron-dependent oxygenase (IPR005123) is the most promising, since it has a higher expression ratio following exposure to 50 μM TBT. All the listed genes are presumed to deliver better results for higher TBT concentration, since their expression ratios are always higher for higher TBT concentrations. However, following the construction of the bioreporter, further studies are necessary to assess its sensitivity and specificity.

FINAL REMARKS

13. Conclusion

The transcriptome of *A. molluscorum* Av27 was sequenced, allowing the identification of some genes that seem to be involved in TBT resistance and degradation. Likewise, it allowed for the identification of genes that can be used as bioreporters of TBT exposure (Figure 15).

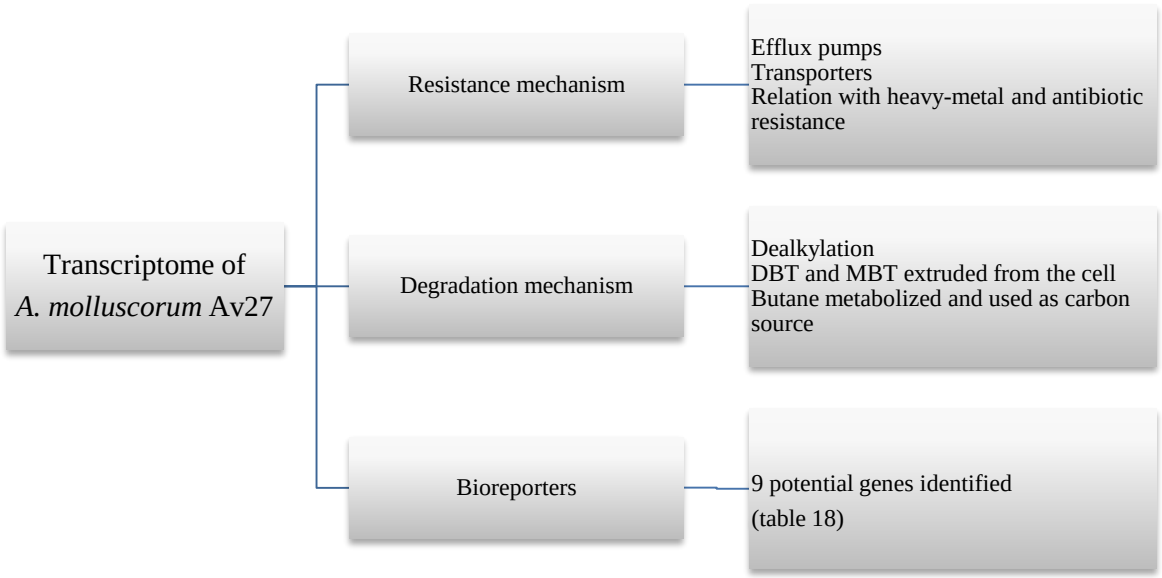


Figure 15 – Overview of the major conclusions of this study.

In this study, some efflux pumps, transporters and other proteins involved in resistance to antibiotics and heavy metals were shown to be over-expressed in the presence of TBT. The relation between these resistance mechanisms was previously suggested by other authors (Suzuki, Fukagawa et al. 1992; Fukagawa, Konno et al. 1994; Jude, Arpin et al. 2004; Hernould, Gagné et al. 2008) and was also confirmed in the present study. A relation between heat-shock proteins and TBT resistance was also suggested based on the analysis of the transcriptome of *A. molluscorum* Av27.

Overall, these results suggest that, in *A. molluscorum* Av27, the TBT resistance/degradation is complex and results from the interplay of several proteins, mainly efflux pumps and other transporters.

Many proteins with unknown function that are significantly over-expressed following exposure to TBT (IPR007497, IPR009809, IPR016596, IPR002549, IPR005358,

among others) were identified. Nonetheless, it was not possible to undoubtedly associate any of those proteins to any specific function.

Despite the progress and contribution of this study, it was not possible to unveil the mechanism behind TBT resistance/degradation. Nevertheless, taking into account the information gathered from this and from other studies, a mechanism for TBT resistance/degradation in *A. molluscorum* Av27 can be proposed based on the mechanism advanced by Cruz, Caetano et al. (2007): TBT is probably captured into siderophore-like structures, where it is degraded through dealkylation into DBT and MBT. These less toxic compounds are then extruded from the cell, probably through efflux pumps as, for instance, SugE and TolC. Another product resulting from TBT degradation is butane, which is probably metabolized in the cell and used as carbon source (Figure 16).

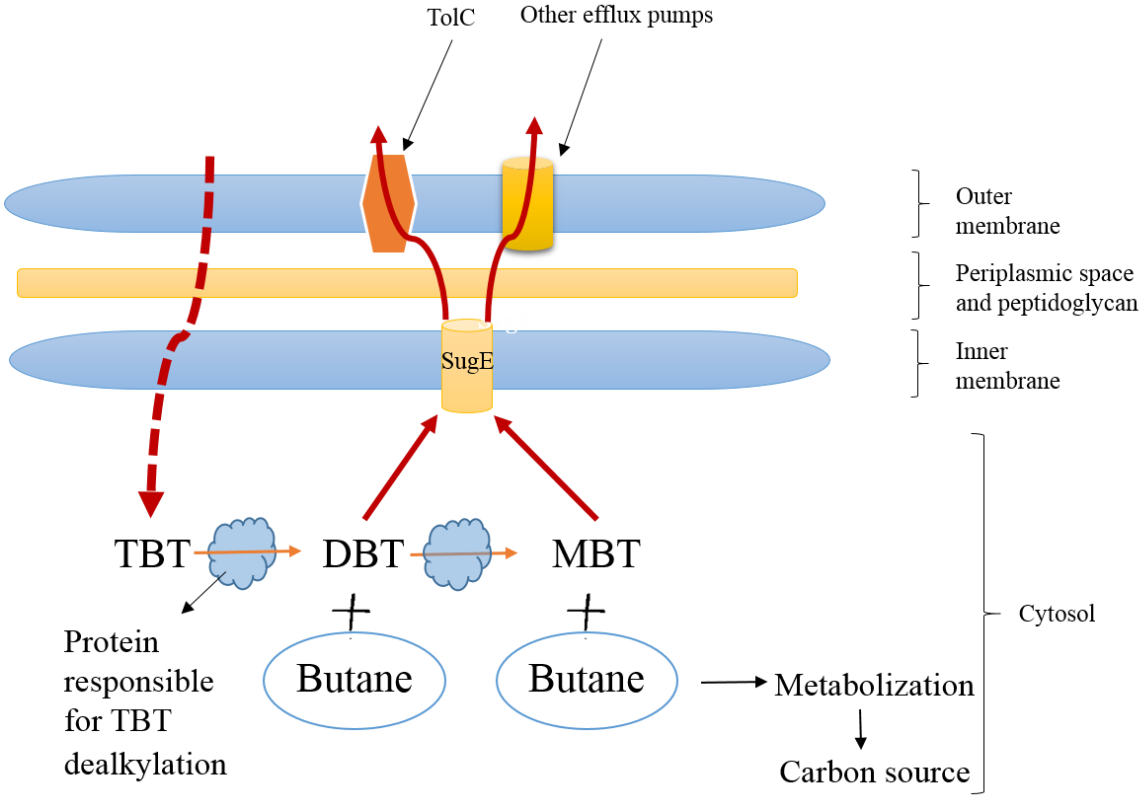


Figure 16 - Proposed mechanism for TBT degradation in *A. molluscorum* Av27.

Additionally, some genes that can be used to develop bioreporters for TBT were also identified.

Since the genome of *Aeromonas molluscorum* Av27 is not available yet, the transcriptomic data herein provided are a valuable resource for comparative genome

analysis. Furthermore, this study surely augmented the knowledge of the functional genomic basis of the TBT resistance and degradation mechanism.

14. Future perspectives

The analysis of the transcriptome of *A. molluscorum* Av27 revealed some proteins with unknown function which were differentially expressed in the presence of TBT. The annotation of these proteins is of great importance, since it could provide relevant information that can shed more light on the mechanism under investigation. In fact, some of these proteins may be involved in the breakdown of the Sn-C bond, thus degrading TBT into DBT and MBT.

The genes with potential to be used as bioreporters must be further studied, in order to confirm their usefulness as sensor elements.

Although this study has contributed to the comprehension of the mechanism of resistance/ degradation of TBT, more studies are required to fully clarify these somehow complex mechanisms.

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APPENDIX

Expression levels

Table A 1 - Some relevant over-expressed and under-expressed genes in *Aeromonas molluscorum* Av27 following TBT exposure. Green: over-expressed genes (expression ratio ≥2); red: under-expressed genes (expression ratio <0.5).

Interpro	Interpro Description	e-value	p-value	Ratio (5 µM TBT)	Ratio (50 µM TBT)	Gene Ontology
IPR000119	Histone-like bacterial DNA-binding protein	2.01E-37	>0.05	1	7	DNA binding
IPR000123	RNA-directed DNA polymerase (reverse transcriptase), msDNA	1.08E-07	1.09310E-03	0.448	0.345	RNA-directed DNA polymerase activity
IPR000214	Zinc finger, DNA glycosylase/AP lyase-type	1.69E-38	>0.05	0.800	0.200	DNA repair
IPR000297	Peptidyl-prolyl cis-trans isomerase, PpiC-type	0.000	1.71815E-12	8	25	Isomerase activity
IPR000298	Cytochrome c oxidase, subunit III	5.08E-33	>0.05	0.182	0.909	Heme-copper terminal oxidase activity
IPR000390	Small multidrug resistance protein family	1.08E-14	3.12892E-03	0.129	0.032	Stress response
IPR000473	Ribosomal protein L36	1.10E-09	>0.05	0.435	0.522	Translation

IPR000835	Transcription regulator HTH, MarR	1.57E-04	6.49251E-11	0	7.500	Sequence-specific DNA binding transcription factor activity
IPR000866	Alkyl hydroperoxide reductase subunit C/ Thiol specific antioxidant	0.000	>0.05	0.500	0.500	Oxidation-reduction
IPR000883	Cytochrome c oxidase, subunit I	0.000	>0.05	0.250	1	Cytochrome-c oxidase activity
IPR001023	Heat shock protein Hsp70	0.000	1.54143E-43	0.810	2.857	Stress response
IPR001036	Acriflavin resistance protein	0.000	1.37998E-07	0	3.125	Transporter activity
IPR001140	ABC transporter, transmembrane domain	0.000	>0.05	2	1	Transporter activity
IPR001327	Pyridine nucleotide-disulphide oxidoreductase, NAD-binding domain	8.20E-18	0.00000E+00	4	124	Oxidation-reduction
IPR001404	Heat shock protein Hsp90	0.000	4.16532E-05	1.333	6.667	Stress response
IPR001433	Oxidoreductase FAD/NAD(P)-binding	3.81E-15	>0.05	0	0	Oxidation-reduction
IPR001460	Penicillin-binding protein, transpeptidase	0.000	>0.05	2.750	0.750	Penicillin binding

IPR001623	Heat shock protein DnaJ, N-terminal	0.000	6.46528E-04	5	13	Stress response
IPR001790	Ribosomal protein L10/acidic P0	0.000	1.70626E-06	0.441	0.487	Translation
IPR001844	Chaperonin Cpn60	0.000	1.83579E-22	0.806	4.032	Protein folding
IPR001848	Ribosomal protein S10	0.000	1.69024E-09	0.626	0.242	Translation
IPR001854	Ribosomal protein L29	1.697E-18	>0.05	0.396	0.415	Translation
IPR002059	Cold-shock protein, DNA-binding	5.57E-23	1.31077E-07	0.140	0.262	Stress response
IPR002068	Heat shock protein Hsp20	3.22E-41	1.28001E-18	0.281	2.875	Stress response
IPR002150	Ribosomal protein L31	2.51E-23	>0.05	0.200	0.267	Translation
IPR002429	Cytochrome c oxidase subunit II C-terminal	0.000	5.88185E-09	0.333	0	Cytochrome-c oxidase activity
IPR002482	Peptidoglycan-binding Lysin subgroup	0.000	1.53444E-14	2	36	Cell wall macromolecule catabolic process
IPR002523	Mg2+ transporter protein, CorA-like/Zinc transport protein ZntB	3.81E-26	>0.05	10	3	Metal ion transmembrane transporter activity
IPR002524	Cation efflux protein	2.89E-34	>0.05	0.167	1.333	Cation transmembrane transporter activity

IPR002549	Uncharacterised protein family UPF0118	1.454E-27	9.31319E-04	0	8	Unknown
IPR002586	Cobyrinic acid a,c-diamide synthase	6.75E-05	2.82816E-15	0.144	0.225	Catalytic activity
IPR002771	Multiple antibiotic resistance (MarC)-related	0.000	>0.05	3.500	0	Stress resistance
IPR003033	SCP2 sterol-binding domain	8.99E-22	>0.05	0.250	1	Sterol binding
IPR003423	Outer membrane efflux protein TolC	0.000	1.20770E-20	3.080	6.200	Transporter activity
IPR003439	ABC transporter-like	0.000	>0.05	0.182	0.273	Transporter activity
IPR003787	Sulphur relay, DsrE/F-like protein	7.89E-19	>0.05	0	0	Oxidation-reduction
IPR004088	K Homology, type 1	0.000	5.05344E-25	0.273	0.136	RNA binding
IPR004161	Translation elongation factor EFTu/EF1A, domain 2	0.000	>0.05	0.429	0.143	Translation
IPR004323	Divalent ion tolerance protein, CutA1	3.00E-18	1.19775E-02	8.500	0	Stress response
IPR004360	Glyoxalase/bleomycin resistance protein/dioxygenase	0.000	0.00000E+00	0.095	2.476	Catalytic activity
IPR004491	Heat shock protein HslU	0.000	>0.05	5	2	Stress response

IPR004565	Outer membrane lipoprotein LolB	1.24E-23	3.51631E-04	0	9	Protein transport
IPR004658	Outer membrane lipoprotein Slp	1.05E-10	>0.05	0	0	Stress response
IPR004670	Na+/H+ antiporter NhaA	0.000	1.20260E-12	0.187	0	Sodium ion transport
IPR005123	Oxoglutarate/iron-dependent oxygenase	3.97E-12	5.58005E-19	2	46	Oxidation-reduction
IPR005358	Uncharacterised protein family UPF0153	0.000	3.65728E-39	0	85	Unknown
IPR005480	Carbamoyl-phosphate synthetase, large subunit, oligomerisation	0.000	1.13391E-02	3	8	Catalytic activity
IPR006016	UspA	0.000	1.10400E-10	0.422	0.339	Stress response
IPR006121	Heavy metal-associated domain, HMA	1.33E-31	3.84074E-03	0.250	1.375	Metal ion transport
IPR006660	Arsenate reductase-like	1.25E-20	1.31484E-04	0	10	Stress response
IPR007210	ABC-type glycine betaine transport system, substrate-binding domain	0.000	0.00000E+00	0.115	0.043	Transporter activity
IPR007267	GtrA-like protein	0.000	1.79538E-05	0	12	Polysaccharide biosynthetic process

IPR007329	FMN-binding	5.32E-36	1.30536E-05	2	15	FMN binding
IPR007420	Protein of unknown function DUF465	3.09E-07	1.69502E-02	0.420	0.957	Unknown
IPR007497	Protein of unknown function DUF541	3.732E-39	8.09826E-03	4.500	7.500	Unknown
IPR007863	Peptidase M16, C-terminal	1.13E-05	1.85082E-14	0	34	Catalytic activity
IPR008269	Peptidase S16, Lon C-terminal	0.000	>0.05	0.022	0.200	Catalytic activity
IPR008991	Translation protein SH3-like	0.000	>0.05	0.324	0.441	Translation
IPR009056	Cytochrome c domain	3.49E-12	6.56960E-06	0	13	Electron carrier activity
IPR009057	Homeodomain-like	4.63E-24	1.70532E-02	0.460	0.360	DNA binding
IPR009081	Acyl carrier protein-like	2.19E-08	>0.05	0	0.167	Synthesis of fatty acids
IPR009809	Protein of unknown function DUF1379	1.78E-13	1.01796E-05	0	14	Unknown
IPR010879	Domain of unknown function DUF1508	1.12E-30	>0.05	2.231	2.538	Unknown
IPR010920	Like-Sm ribonucleoprotein (LSM)-related domain	0.000	>0.05	0.267	0.133	Modulators of RNA biogenesis
IPR011008	Dimeric alpha-beta barrel	9.16E-04	>0.05	0	2.500	Catalytic activity
IPR011032	GroES-like	7.71E-41	1.71186E-03	12.333	36	Protein folding

IPR011322	Nitrogen regulatory PII-like, alpha/beta	3.00E-18	1.19775E-02	8.500	0	Stress response
IPR011703	ATPase, AAA-3	2.75E-10	>0.05	0.250	0	Catalytic activity
IPR012338	Beta-lactamase/transpeptidase-like	1.27E-25	>0.05	0	0	Stress response
IPR012394	Aldehyde dehydrogenase NAD(P)-dependent	4.82E-29	9.31319E-04	0	8	Oxidation-reduction
IPR013116	Acetohydroxy acid isomeroreductase, catalytic	1.40E-42	1.17829E-03	1	9	Ketol-acid reductoisomerase activity
IPR014729	Rossmann-like alpha/beta/alpha sandwich fold	0.000	1.10400E-10	0.422	0.339	Catalytic activity
IPR016596	Uncharacterised conserved protein UCP012335	1.23E-16	7.55141E-03	1	4	Unknown
IPR019545	DM13 domain	4.84E-28	4.01264E-08	0	18	Oxidation-reduction