Impact of Environmental Conditions on Expression Levels of Resistance-Nodulation-Division (RND) Efflux Pumps and Outer Membrane Porins in *Acinetobacter baumannii*

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In Partial Fulfillment of the Requirements

For the Degree of

Master of Science in Applied Bioscience

University of Ontario Institute of Technology

Oshawa, Ontario

September 2012

ABSTRACT

Acinetobacter baumannii is a globally emerging Gram-negative pathogen accounting for a variety of nosocomial conditions, including pneumonia, meningitis, peritonitis, urinary tract infections and, in particular, wound infections in soldiers. One of the major reasons for *A. baumannii* success as a human pathogen is attributed to a tremendous potential of this organism for development of the multidrug resistant phenotype (MDR). This organism's ability to regulate expression of its intrinsic antibiotic resistance determinants, such as Resistance-Nodulation-Cell Division (RND) efflux pumps and outer membrane proteins, is regarded to be amongst the most important strategies in the arsenal of this pathogen. Despite considerable efforts being dedicated in the attempt to understand the underlying mechanisms in the development of the MDR phenotype in *A. baumannii*, the role of environmental stress factors is often overlooked. Evidence from other Gram-negative pathogens suggests that adaptive responses of the bacterial cell to environmental stress often target the same cellular processes that are exploited by antibiotics, therefore indirectly promoting the emergence of the MDR phenotype. This study shows the impact of a variety of environmental factors, namely iron(III) availability, oxidative stress, salicylate exposure, steady-state osmotic stress, ethanol shock, exposure to different incubation temperatures and exposure to blue light on expression levels of three RND efflux pumps and three outer membrane porins of *A. baumannii*. In addition, we investigated the difference in expression levels of the selected two-component systems between a type strain of *A. baumannii*, ATCC 19606, and two clinical isolates with the MDR and biofilm-forming phenotypes. The quantitative-real time polymerase chain reaction analysis revealed that levels of iron(III) in growth medium, oxidative stress and ethanol shock were amongst the most potent factors capable of influencing expression of RND efflux pumps and outer membrane porins.

ACKNOWLEDGEMENTS

I would like to thank everyone who helped me in my research, providing helpful guidance and encouragement along the way. Especially, to my supervisor, Dr. Kumar, who proved to be more than just a scientific supervisor and has lent his support on numerous and numerous occasions of shattered motivation and other "life situations". My sincerest gratitude to the members of my committee, Dr. Bonetta , Dr. Forrester and Dr. Green-Johnson for their valuable input and patient guidance towards that light at the end of the tunnel.

To my family for all of the support and words of wisdom. To my lab-mates and the extended "4th floor community" of graduate students for the passionate discussions and heated arguments on evolution, religion, economics, precious metals market, politics, conspiracy theories, zombie apocalypse, global warming and so much more.

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List of Abbreviations

ABC	ATP binding cassette
ATP	adenosine triphosphate
Asp	aspartate
BLUF	blue-light-using sensing flavin (domain)
cDNA	complimentary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetate
Fur	ferric uptake regulator
Glu	glutamic acid
His	histidine
HK	histidine kinase
ICU	intensive care unit
kDa	kilodalton
LB	Luria-Bertani (medium)

LOV	light, oxygen or voltage (domain)
MATE	multidrug and toxic compound extrusion
MDA	membrane damaging agents
MDR	multidrug resistant
MHB	Mueller-Hinton broth
MFS	major facilitator superfamily
MFP	membrane fusion protein
mRNA	messenger ribonucleic acid
NRT	no reverse transcriptase
NTC	no template control
OMP	outer membrane protein
PBP	penicillin binding protein
PCR	polymerase chain reaction
PYP	photosensitive yellow protein
qRT	quantitative reverse transcriptase
RND	resistance-nodulation-division
ROS	reactive oxygen species

rpm	rotations per minute
RR	response regulator
SDS	sodium dodecyl sulphate
TCS	two-component system
TE	tris(hydroxymethyl)aminomethane ethylenediaminetetraacetate
UV	ultraviolet
vol/vol, v/v	volume/volume
YPDA	yeast peptone dextrose agar

1. INTRODUCTION

A *ACINETOBACTER BAUMANNII:* CLINICAL RELEVANCE AND ANTIBIOTIC RESISTANCE STRATEGIES

Acinetobacter baumannii is a rapidly emerging opportunistic pathogen in health care settings, targeting immunocompromised and/or critically ill patients and is considered to be one of the most difficult Gram-negative bacilli to eradicate. Infections caused by *A. baumannii* include nosocomial-acquired and/or ventilator-associated pneumonia, meningitis, peritonitis, urinary tract infections, bacteremia, wound infection and, to a lesser extent, endocarditis, endophthalmitis and keratitis (Peleg *et al.*, 2008; Michalopoulos and Falagas, 2010).

It has been reported that from 1986 to 2003 the proportion of Intensive Care Units (ICU) pneumonia attributed to *Acinetobacter* species has increased from 4.0% to 7.0% (Gaynes and Edwards, 2005). Mortality rates associated with *A. baumannii* infections in ICUs vary in different reports: from 40% (Klompas, 2010) to anywhere between 26% and 68% (Maragakis and Perl, 2010).

The success of *A. baumannii* as a hospital pathogen can be attributed to several key factors that include its ability to survive under a wide range of environmental conditions (especially, on dry surfaces of medical instruments and devices) (Klompas, 2010) and, most importantly, due to the organism's tremendous potential for acquisition of antibiotic resistance determinants. It is estimated that in the United States alone, the occurrence of multi-drug resistant (MDR) *A. baumannii* has increased from 6.7% in 1993 to 29.9% in 2004, which is twice the rate of MDR emergence of any other ICU-relevant Gram-negative bacillus (Hood *et al*., 2010).

The variety of antibiotic resistance strategies employed by *A. baumannii* can be classified into 3 major categories (Peleg *et al.,* 2008) such as: 1) production of antibiotic-inactivating

enzymes (e.g., β-lactamases, carbapenemases, cephalosporinases); 2) alterations that change the antibiotic's target or the bacterial cell functions (e.g., downregulation of affinity to and/or expression of penicillin-binding proteins, PBPs; mutations in the *gyrA* and *parC* topoisomerase enzymes, quinolone targets); 3) precluding the antibiotic's access to its cell target (e.g., downregulation of porin channels and other outer membrane proteins' expression). These three mechanisms are by no means mutually exclusive and are considered to be functioning together in clinically relevant MDR *A. baumannii* strains.

B RESISTANCE-NODULATION-DIVISION EFFLUX PUMPS OF *A. BAUMANNII*

1. Structure

One of the ways for *A. baumannii* to prevent access of antibiotic to its cellular target is the production of efflux pumps located in the bacterial cell wall. These are the multi-subunit proteins that are responsible for efflux of the agents that can potentially compromise the integrity of bacterial cell membrane (Bonomo and Szabo, 2006), as well as various classes of antibiotics (e.g., β-lactams, carbapenems, cephalosporins, aminoglycosides). Efflux pumps belong to the Resistance-Nodulation Cell Division (RND) superfamily of drug transporters, which represent a tripartite complex comprised of an outer membrane protein (OMP), an inner membrane RND transporter and a membrane fusion protein (MFP) that links the OMP and RND components together. RND drug transporters are usually encoded by chromosomal genes (rarely by plasmids), and are considered to be the most relevant family of efflux pumps in Gram-negative bacteria. RND pumps are driven by the substrate/H^+ antiport mechanism and have been linked not only to efflux of antibiotics, but also to efflux of a broad range of structurally unrelated

molecules/ions such as biocides, heavy metals, dyes, organic solvents, detergents, metabolic inhibitors, bile salts and homoserine lactones involved in quorum sensing (Poole, 2004).

Three RND efflux pumps have been described in *A. baumannii* to date: AdeABC, AdeIJK and AdeFGH. All three pumps have the regulatory sequences located either upstream of the structural genes (in the case of AdeABC and AdeFGH) of these pumps (Coyne *et al*., 2010; Coyne and Perichon, 2010) or randomly in the genome as seen for the *adeN* regulator gene of the AdeIJK efflux pump (Rosenfeld *et al.,* 2012).

2. AdeABC RND efflux pump

a. *Clinical significance of AdeABC pump*

AdeABC is, arguably, the most important RND efflux pump in MDR clinical isolates of *A. baumannii*. It is found in 80% (53% to 97%, varying among different reports (Gaynes and Edwards, 2005; Peleg *et al.*, 2008)) of clinical isolates of *A. baumannii* and is known to confer high levels of resistance to numerous groups of antimicrobials: β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, macrolides, chloramphenicol, trimethoprim, as well as tigecycline (a glycylcycline family antibiotic often regarded as a "drug of last resort" (Magnet *et al*., 2001, Rosenfeld *et al*., 2012). AdeABC is thought to play a role in resistance to yet another clinically very important class of antibiotics, the carbapenems, even though further research is required to confirm this (Coyne *et al.,* 2010). The clinical relevance of the AdeABC efflux pump is further highlighted by the fact that it seems to be predominantly present in clinical isolates but could not be detected in 32 environmental isolates of *Acinetobacter baumannii* (Huys *et al*., 2005).

b. AdeABC regulation

Two genes immediately upstream of the *adeA* gene have been identified, *adeR* and *adeS.* These genes encode a response regulator and a sensor kinase, respectively, which are a part of a two-component regulatory system (Marchand *et al.,* 2004). TCS are signal transduction pathways in bacteria (as well as certain Archaea, protozoa, plants and fungi) and respond to changes in environmental conditions. Disruption of the *adeR* and *adeS* genes by insertion mutagenesis results in increased susceptibility of the mutant strains of *A. baumannii* to aminoglycosides and other substrates of the AdeABC pump (Marchand *et al*., 2004; Rosenfeld *et al*., 2012), indicating that *adeR* is a positive regulator of the AdeABC pump expression. A point worth mentioning is that the observed behavior of the AdeRS two-component system as a transcriptional activator is uncommon, as most regulatory two-component systems described in bacteria so far have been reported to be transcriptional repressors (Marchand *et al*., 2004; Mizuno and Mizushima, 1990). Two-component systems have been reported to control the expression of various drug efflux pumps (e.g., MdtABC and YhiUV in *Escherichia coli* (Nagakubo *et al*., 2002), RagCD in *Bradyrhizobium japonicum* (Lindermann *et al*., 2010) and SmeABC in *Stenotrophomonas maltophilia* (Chang *et al*., 2004) but overall are considered to be an uncommon mode of regulation of efflux pumps (Marchand *et al*., 2004).

3. AdeIJK RND efflux pump

The AdeIJK efflux pump is known to impart intrinsic resistance to such important classes of antibiotics as fluoroquinolones, tetracyclines, lincosamides, cephalosporins and β-lactams (Damier-Piolle *et al.,* 2008*)*. It also contributes to active efflux of tigecycline, rifampin, chloramphenicol, co-trimoxazole, novobiocin and fusidic acid. Importantly, aminoglycosides

have not been shown to be substrates of AdeIJK. AdeN has been recently identified as a pump repressor from the TetR family of transcriptional regulators (Rosenfeld *et al*., 2012). Besides antimicrobials, both AdeABC and AdeIJK are involved in active efflux of biocides, detergents, antiseptics and dyes (Damier-Piolle *et al*., 2008; Magnet *et al*., 2001; Rajamohan *et al*., 2010).

4. AdeFGH RND efflux pump

The AdeFGH RND efflux system, when overexpressed, has been shown to impart resistance to a number of antibiotic families: fluoroquinolones, chloramphenicol, trimethoprim, clindamycin; and to a lesser extent to tetracyclines, tigecycline and sulfamethoxazole (Coyne and Perichon, 2010). This pump is known to be under control of the AdeL protein, a LysR-family regulator, encoded immediately upstream of the *adeFGH* operon (Coyne *et al*., 2010). AdeFGH is believed to play a role in the MDR phenotype when overexpressed, commonly due to mutations in the AdeL regulator, and is not known to be constitutively expressed in wild-type strains of *A. baumannii* (Coyne *et al*., 2010).

C TWO-COMPONENT SYSTEMS

The regulatory elements, such as the previously mentioned *adeR* and *adeS* genes, known to regulate transcription from the *adeABC* operon, belong to a class of so called two-component signaling systems that are extremely diverse and abundant among prokaryotes. Such systems are believed to elicit control over almost virtually all functions and responses of a bacterial cell to both endogenous and exogenous stimuli (Wuichet *et al*., 2010; Whitworth and Cock, 2009). The basic organization of these signaling systems involves one gene encoding a histidine kinase protein (HK), capable of autophosphorylation, and a response regulator protein (RR) that is,

essentially, an effector protein that can accept a phosphate group from the autophosphorylated sensor kinase protein. Upon activation, the response regulator goes on to activate other proteins involved in a signal transduction pathway or serves as a transcription factor. Structural organization of the histidine kinase and the response regulator involves two distinct domains on each protein: sensory (or input) and transmitter domains for the histidine kinase and receiver and regulatory (or output) domains for the response regulator protein. Importantly, the sensory domain of the histidine kinase can be both extracellular and cytoplasmic (Williams and Whithworth, 2010; Wuichet *et al*., 2010; Koretke *et al*., 2000).

The transfer of the phosphate group between sensors and regulators, a phosphorelay, occurs between the transmitter domain of the sensor kinase (usually on its C-terminus) and a receiver domain (N-terminus) of the response regulator protein. The transmitter domain of the sensor kinase and the receiver domain of the response regulator contain highly conserved His and Asp residues, respectively, directly involved in a phosphorelay within a signaling transduction system (Wuichet *et al*., 2010; Koretke *et al*., 2000). This basic organization of a two-component system can be more complex, however, with both the sensor kinase and the response regulator having more than one target or both proteins having multiple sensory, transmitter, receiver or regulatory domains. Examples of the intermediate components between the sensor kinase and response regulator proteins have also been described (Williams and Whithworth, 2010). These usually serve the function of diverting and extending the phosphorelays associated with a particular two-component system.

The two-component systems have been found in all three domains (Bacteria, Archaea and Eukarya),were identified in 864 out of 899 fully sequenced bacterial genomes (Wuichet *et al*., 2010) and, interestingly, are lacking only in pathogens with severely reduced genomes (e.g.,

Mycoplasma spp.) due to their parasitic lifestyle. Only 50% of the Archaea and fewer than 30% of Eukarya genomes have been found to contain two-component systems. Importantly, within the domain Eukarya, no TCS could be identified in metazoan lineages and are predominantly distributed, instead, into fungal and plant lineages (Wuichet *et al*., 2010). Due to such a significant role of TCS in the control of almost all aspects of bacterial behavior and their preferential distribution in bacteria, sensor kinases could be targeted by new antimicrobials.

D OUTER MEMBRANE PROTEINS IN *A. BAUMANNII*

The increased efflux of antimicrobial agents from the cytoplasm, associated with the activity of RND efflux pumps, can be accompanied by a decreased influx of antibiotics into the cytoplasm. The latter is usually associated with the loss of outer membrane porins from the bacterial cell wall (Peleg *et al*., 2008). Very little is known regarding the role of outer membrane porins in *A. baumannii* with respect to their role in antibiotic resistance. So far, the loss of outer membrane porins, namely CarO, 33kDa-porin and OprD, has been linked to a decreased susceptibility of *A. baumannii* only to carbapenemases (Mar Tomas *et al*., 2005; Catel-Ferreira *et al*., 2011; Mussi *et al*., 2007; Siroy *et al*., 2005; Catel-Ferreira *et al*., 2012). No reports of decreased outer membrane protein expression leading to a decreased susceptibility of *A. baumannii* to any other classes of antimicrobials have been published to date.

1. CarO outer membrane protein

The CarO outer membrane protein is the best characterized porin in *A. baumannii*. CarO has been shown to contain two polypeptide chains within its structure, namely CarOa and CarOb (Catel-Ferreira *et al*., 2011). Both of these polypeptides were shown to be capable of forming

narrow channels with both of them possessing certain affinity and specificity towards imipenem, a major clinically relevant carbapenem currently in use. Interestingly, CarOb was found to be significantly more specific towards imipenem than CarOa (Catel-Ferreira *et al*., 2011). Conversely, both polypeptides were found to lack the binding site to meropenem, yet another major carbapenem on the market. The loss of CarO porin in clinical isolates of *A. baumannii* has also been linked to a decrease in susceptibility to carbapenems (Mar Tomas *et al*., 2005; Catel-Ferreira *et al*., 2011; Mussi *et al*., 2007; Siroy *et al*., 2005).

2. OprD outer membrane protein

OprD is a 43-kDa porin that shows 49% similarity to the OprD porin of *Pseudomonas aeruginosa* (Dupont *et al*., 2005), which is known to form an 18-β-stranded barrel and a narrow channel within itself. Interestingly, the channel is lined with basic amino acids but has been shown to contain a negatively charged pocket in the periplasm that is believed to be involved in the uptake of positively charged substrates (Dupont *et al*., 2005). In *P. aeruginosa,* OprD has been shown to be involved in the transport of carbapenems (Trias and Nikaido, 1990) as well as positively charged amino acids (Hancock and Brinkman, 2002). Characterization of the OprD porin in *A. baumannii* has revealed, however, that this porin could be functionally closer to the OprQ family of porins of *P. aeruginosa*. This suggests that the OprD homologue in *A. baumannii* may not be involved in the transport of carbapenems across the cell membrane, as previously thought, and, hence, is unlikely to play any role in resistance to this class of antimicrobials (Catel-Ferreira *et al*., 2012). The latter group has suggested, instead, that the OprD porin may play a role in iron(III) and magnesium ion uptake under stressful conditions, as well as in the cell adhesion, biofilm formation and host colonization; making this porin an important virulence factor in *A. baumannii*.

3. 33-kilodalton outer membrane protein

A homologue of the 33-kDa outer membrane protein has not been characterized in *A. baumannii*. The very little that is known about this porin is the evidence that a loss of the 33- to 36-kilodalton outer membrane protein imparts carbapenem resistance in *A. baumannii* (Mar Tomas *et al*., 2005; Clark, 1996; Bou *et al*., 2000; Costa *et al*., 2000). By analogy with the CarO and OprD porins, these reports hint at 33-kDa porin's role in the transport of carbapenems into the bacterial cell.

E ENVIRONMENTAL STRESS FACTORS AND ANTIBIOTIC RESISTANCE

The *in vivo* methods of modification of antibiotics leading to decreased efficacy of the antimicrobial agents discussed above are typically employed upon direct encounter of the bacterial cell with antibiotics. In addition, a bacterial cell can end up acquiring resistance to antimicrobials as a consequence of the protective and/or adaptive response to antibiotic unrelated factors (Poole, 2012; Ruiz and Silhavy, 2005). The majority of external stress factors that a bacterial cell encounters in the environment (and even more so upon exposure of the pathogen to the host environment), generally interfere with bacterial growth and proliferation. The protective and/or adaptive responses employed by the bacterial cells usually target and interfere with the same major cellular processes and components that are being targeted by clinically relevant antibiotics (e.g., efflux systems and outer membrane porins, mutational rate, cell envelope stability, horizontal gene transfer) (Poole, 2012; Lee *et al*., 2009). Quite often, the general stress response of the bacterial cell involves activation and recruitment of a number of general transcription factors (e.g., RpoE, σ^E, in *E. coli* (Ruiz and Silhavy, 2005); AlgU in *P. aeruginosa*

(Fraud *et al*., 2008) and/or two-component systems (e.g., AmgRS in *P. aeruginosa* (Lee *et al*., 2009); CpxRA in *E. coli* (Ruiz and Silhavy, 2005). Hence, in this way the environmental stresses may indirectly select for the development of the antibiotic resistant phenotypes in clinically relevant pathogens. For example, Mg^{2+} limitation has been shown to promote resistance to powerful cationic antimicrobials such as polymyxin B that is often regarded as "drugs of last resort" in *P. aeruginosa* (Macfarlane *et al*., 1999) and *Salmonella enterica* (Groisman *et al*., 1997).

1. Fe^{3+} availability and RND efflux pumps and outer membrane porins expression

Iron-depleted conditions are well-known to be utilized by bacteria as a signal of infecting the host. This strategy involves the transcriptional control of iron(III)-regulated genes by a ferric uptake regulator protein (Fur). The Fur protein is known to act as an iron(III)-dependent repressor which targets promoters of the iron(III)-regulated genes, repressing these genes under high iron(III) availability conditions. Repression of the iron(III)-regulated promoters by the Fur protein is lifted when iron(III) becomes scarce in the extracellular media. The orthologs of the *fur* gene have been characterized in *A. baumannii* (Daniel *et al*., 1999), as well as in a number of other bacterial species (e.g., *Yersinia, Salmonella, Helicobacter pylori, Pseudomonas*, Escolar *et al*., 1999). Interestingly, most Fur orthologs can complement the *E. coli fur* mutants, which suggests that the molecular mechanism involved in the control of iron(III)-regulated genes is highly conserved among a variety of bacterial species. Besides the transcriptional control of the genes directly involved in iron(III) metabolism, the Fur protein has been also shown to be involved in the control of the genes that are a part of the bacterial cell's response to oxygen radicals, acid shock, as well as the genes involved in chemotaxis and production of toxins and

virulence factors (Escolar *et al*., 1999). The link between the Fur repressor in the regulation of RND efflux pump expression, however, has not been established to date.

2. Osmolarity and RND efflux pumps and outer membrane porins expression

Elevated osmotic pressure outside the bacterial cell is one of the major environmental stress factors that pathogens can be exposed to in the host. Very scarce data, however, is available regarding the response of *A. baumannii* to the stress factors that it can be exposed to upon infection of the host.

A change of concentration of various solutes in the media surrounding the bacterial cell leads to a short-lived imbalance between external and internal turgor pressure. This, in its turn, causes the flux of water molecules across the cell membrane; a cellular process that is responsible for changes in the cellular shape, organization and even chemical composition that may lead to a physiological response (Wood, 1999). The osmosensing "machinery" of the bacterial cell is comprised of chemosensors and osmosensors. The chemosensory mechanisms of the bacterial cell are based on the stereospecificity of ligand-receptor interactions that allow the cell to detect changes in the solute concentrations both inside and outside of the cell. The osmosensors, however, are usually the macromolecules that respond with a conformational change to changes in the solute composition and/or its concentration as well as to mechanical stimuli associated with osmolarity fluctuations (Wood, 1999).

The general effect of osmolarity on transcriptional regulation can be explained from an angle of DNA-protein interactions due to direct involvement of water molecules at the DNA-protein (often an enzyme) interface (Robinson and Sligar, 1994). The role of the water molecules in transcriptional control in the bacterial cell can be illustrated by the Fur protein, a key repressor

involved in the bacterial stress response to iron(III) starvation. The Fur binding to its target DNA sequences and, hence, the effective repression of its regulon, relies on the binding of an Fe(III) ion to its binding site within the Fur protein which causes the subsequent dimerization of the Fur protein and its attachment to DNA. The iron(III) binding site is composed of five amino acids: two Asp, two His and a single Glu residues. A water molecule, however, is required to stabilize Fe(III) binding to the Fur protein in an octahedral arrangement (Miethke and Marahiel, 2007). Any major changes in osmolarity are, hence, capable of interfering with the effectiveness of the Fur protein binding to its target DNA sequences, potentially causing disruptions in its transcriptional control function (Wood, 1999; Robinson and Sligar, 1994).

Another potentially major factor responsible for the transcriptional impact of the osmolarity changes is associated with macromolecular crowding and confinement inside of the bacterial cell. This may play a significant role in weak interactions between macromolecules (such as the interactions between proteins and DNA, for example), especially, given that the macromolecular fraction inside of the cell is known to occupy as much as 50% of the cytoplasmic volume (Wood, 1999). In addition, Minton *et al*. (1992) have shown experimentally that macromolecular crowding, enhanced upon the osmolarity shifts, has a much greater effect on the interactions between macromolecules than on the interactions between macromolecules and small molecules.

3. Temperature and RND efflux pumps and outer membrane porins expression

The effect of temperature on the expression levels of the RND efflux systems in *A. baumannii* has not been described in the literature to date. However, several studies conducted on *E. coli* have established that *E. coli* showed the multidrug resistant phenotype under 37˚C but not under 30˚C (Alekshun and Levy, 1997). Similarly, Hartog *et al*. (2008) reported that the

levels of transcription from the *marRAB* regulatory operon and from the *acrAB* RND efflux operon in *Salmonella enterica* were significantly greater at 37˚C in comparison to 30˚C. Importantly, the aforementioned MarA protein is known to cause the upregulation of expression of the major clinically relevant efflux systems such as AcrAB, for example, in *E. coli, S. enterica* and some other Gram-negatives (Alekshun and Levy, 1997). Therefore, based on these studies, we think there is a reason to believe that an increase in the incubation temperature up from the average room temperature in clinical settings to 37˚C or even higher temperatures (~42˚C), can play a role in the transcriptional upregulation of the genes encoding the RND efflux pumps in *A. baumannii* sp, possibly contributing to the emergence of MDR strains.

4. Salicylate exposure and RND efflux pumps and outer membrane porins expression

Salicylate has been shown to be capable of inducing resistance to several antibiotics in *E. coli* such as chloramphenicol, ampicillin and tetracycline (Cohen *et al*., 1993). The mechanism behind the induction of antibiotic resistance by salicylate was investigated from the perspective of activation of the *marRAB* operon and Cohen *et al*. were able to show up to a 30-50 fold increase in transcription from the *marRAB* operon in the *mar* mutants of *E. coli* treated with sodium salicylate. The antibiotic resistance of the *mar* mutants (e.g., fluoroquinolones and chloramphenicol resistance) is believed to be achieved through a combination of factors, among which the combination of increased active efflux and decreased cell influx have been suggested to be the major ones. As it was mentioned previously, the *marRAB* operon is known to be involved in the mediation of multi-drug resistance in *E. coli*, as well as in the regulation of expression of some of the major RND efflux systems (e.g., AcrAB (Alekshun and Levy, 1997) via the transcriptional activator MarA (encoded by the *marRAB* operon). Interestingly, the data obtained by Cohen *et al.* (1993) suggests that the induction of multidrug resistance in *E. coli* by

salicylate is not occurring solely via the *mar* operon activity. To explain this, the authors suggested that alternative routes leading to antibiotic resistance in *E. coli* treated with sodium salicylate exist (Cohen *et al*., 1993).

5. Oxidative stress and RND efflux pumps and outer membrane porins expression

Bacteria are continuously exposed to reactive oxygen species (ROS) when inside of the host. Sources of oxidative stress are both endogenous, due to intrinsic biochemical metabolic reactions, as well as exogenous - primarily due to bacterial cell interaction with the host immune system. Endogenous ROS can be generated as a by-product of reactions involving oxidation of glucose or exposure to UV light (Kinnula *et al*., 1992). The main exogenous source of oxidants and hydrogen peroxide is associated with an encounter of the pathogen with host phagocytes. These host immune cells generate hydrogen peroxide via a series of biochemical reactions that are initiated by an increase in oxygen consumption by a phagocyte upon encounter with the pathogen, eventually leading to conversion of the O_2 molecules to the O_2^- radicals, which, in turn, results in dismutation of the O_2^- radicals to hydrogen peroxide. A wide variety of ROS can be further generated through Fenton reactions involving intracellular Fe^{2+} and Fe^{3+} ions and hydrogen peroxide, either produced endogenously or encountered exogenously (Miller and Britigan, 1997). In addition to phagocytes, prospective pathogen may be exposed to hydrogen peroxide through the interaction with endothelial cells, as well as other oxidative species that are normally produced by endothelial cells as a part of the host response to inflammation (Miller and Britigan, 1997; Kinnula *et al*., 1992).

Other major sources of exogenous ROS and hydrogen specifically, are some of the antibiotics that are routinely used in hospitals. It is well-known now that the bactericidal

antibiotics, namely fluoroquinolones, aminoglycosides and β-lactams, in addition to affecting their molecular targets (topoisomerase, ribosomes and the cell wall, respectively), are also involved in production of ROS that proceed to killing the bacterial cell via macromolecular and organelle damage (Kohanski *et al*., 2007).

6. Ethanol exposure and RND efflux pumps and outer membrane porins expression

Ethanol has been shown to increase *A. baumannii* virulence in different models. For example, co-incubation of *Saccharomyces cerevisiae* and *A. baumannii* has been found to not only promote bacterial growth but also help tolerate the toxic effects of salts (Smith *et al*., 2004). It is noteworthy that the observed effect was specific to ethanol and could not be replicated with methanol or butanol. Elsewhere, it was reported that ethanol-fed *A. baumannii* was more pathogenic towards *Caenorhabditis elegans* (Smith *et al*., 2004). The increased pathogenicity of ethanol-fed *A. baumannii* was subsequently confirmed by Lamarche *et al.* (2008), who used *Dictyostelium discoideum* as a model for pathogen-host interactions. Camarena *et al*. (2010) have also reported the induction of the stress response genes in *A. baumannii* ATCC 17978 strain (permeases and a few efflux pumps) in response to ethanol in growth media. Ultimately, the increased pathogenicity and virulence of *A. baumannii* in the presence of ethanol was attributed to the elevated metabolic capacity as well as to the induction of the stress response genes, namely *groEL* and *hsp90.*

7. Exposure to blue light and RND efflux pumps and outer membrane porins expression

Light has been shown to play a role in pathogenic prokaryotes (Mussi *et al*., 2010). It is believed that light is sensed by the specific light-sensitive receptors, containing the blue-light-sensing-flavin (BLUF) or photoactive yellow protein (PYP), light, oxygen or voltage (LOV)

amongst the most common ones. Several reports have shown that the light response is involved in regulation of a number of bacterial functions with stress response, virulence and biofilm formation being among the most clinically relevant ones (Gaidenko *et al*., 2006; Tschowri *et al*., 2009).

A. baumannii ATCC 17978 is known to encode the N-terminal blue-light-sensing-using flavin (BLUF) domain on an18.6-kDa protein that was shown to regulate *A. baumannii* virulence towards *Candida albicans* as well as the bacterial cell motility and biofilm formation in response to blue light (Mussi *et al*., 2010). No reports linking exposure to blue light to the regulation of expression levels of the RND efflux pumps and/or outer membrane porins in *A. baumannii* or any other Gram-negatives have been published to date.

F RESEARCH OBJECTIVES

HYPOTHESIS:

The protective and/or adaptive responses employed by the bacterial cells in response to the host environment affect the expression of outer membrane porins and resistance-nodulation-division efflux pumps in *A. baumannii* ATCC 19606 and, thus, modulate its antibiotic susceptibility.

The objectives of this thesis are:

- To investigate the impact of some of the major environmental stress factors (incubation temperature, levels of iron(III) availability, oxidative stress, steady-state osmotic stress, salicylate exposure and blue-light exposure) on

transcription of the intrinsic antibiotic resistance determinants, RND efflux pumps and outer membrane proteins, in *Acinetobacter baumannii* ATCC 19606.

- To study the global regulators of antibiotic resistance in *A. baumannii* ATCC 19606 by comparing the expression of the selected two-component regulatory systems in the type strain of *Acinetobacter baumannii*, ATCC 19606, and two clinical isolates of *A. baumannii*, strains AB030 and AB031.

2. MATERIALS & METHODS

A BACTERIAL STRAINS AND GROWTH MEDIUM

A. baumannii ATCC 19606 and two clinical isolates of *A. baumannii*, AB030 and AB031 (Fernando and Kumar, 2011), were used in the study. Bacterial cultures were grown in LB Lennox (10% tryptone, 5% yeast extract, 5% sodium chloride; Biobasic, Markham, Canada) at 37°C with shaking at 200 rpm unless stated otherwise. Mueller-Hinton agar (4% beef infusion solids, 1.5% starch, 17.5% casein hydrolysate, 15% agar; Sigma-Aldrich, Oakville, Canada) was the growth medium used for antibiotic susceptibility assays.

B GROWTH CONDITIONS

A. baumannii ATCC 19606 was subjected to a number of environmental conditions and stresses. Bacterial cultures were subjected to three sets of incubation temperatures: 30°C, 37°C and 42°C. Exposure to salicylate (Biobasic, Markham, Canada) was achieved by adding sodium salicylate to the LB Lennox media to the final concentrations of 1 mM, 2.5 mM and 4 mM. Bacterial cultures were exposed to salicylate from the moment of inoculation of the growth media to the point of collection of the culture for mRNA extraction. The higher concentrations of salicylate, 5 mM and 10 mM, were also tried in the study but the transcription levels of the housekeeping genes tested (16S rRNA-encoding gene and *fmcC*) were found to be significantly fluctuating relative to the baseline conditions (no exogenous salicylate added) making qRT-PCR analysis unreliable.

Exposure to iron(III)-repleted conditions was achieved by adding ferric(III) chloride, $FeCl_3$, (Fisher Scientific, Ottawa, Canada) to a final concentration of 100 μM, while iron(III)-depleted conditions were accomplished by adding a siderophore compound, ferrichrome (Sigma-

Aldrich, Oakville, Canada) to a final concentration of 150 μM in the growth media. Bacterial cultures were exposed to ferric(III) chloride and ferrichrome from the moment of inoculation of the growth media and to the point of collection of the bacterial culture for mRNA extraction.

Ethanol shock was achieved by adding ethanol (Sigma-Aldrich, Oakville, Canada) to the final concentrations of 0.5% and 1.1% (v/v) at OD_{600}=0.6. Bacterial culture was exposed to ethanol for 20 minutes and then harvested for mRNA extraction.

Steady-state hyperosmotic stress was achieved by adding sucrose (Biobasic, Markham, Canada) to the media to the final concentrations of 0.1 M, 0.2 M and 0.3 M. Bacterial culture was exposed to sucrose from the moment of inoculation of the growth media and to the point of collection of the culture for mRNA extraction. The higher concentrations of sucrose, 0.4 M and 0.5 M, were tried in the study but the transcription levels of the housekeeping genes tested (16S rRNA-encoding gene and *fmcC*) were found to be significantly fluctuating relative to the baseline conditions (no exogenous sucrose added) making qRT-PCR analysis unreliable.

The oxidative stress conditions were achieved by adding hydrogen peroxide (Sigma-Aldrich, Oakville, Canada) to the media to the final concentrations of 1 mM and 2 mM at OD_{600}=0.6. Bacterial cultures were exposed to oxidative stress for 20 minutes and then harvested for mRNA extraction. The 20 minutes exposures to ethanol and hydrogen peroxide were performed to ensure that the bacterial cells underwent at least one cell division after exposure to the aforementioned chemical agents and before the cells were harvested for mRNA extraction and qRT-PCR analysis.

C QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE PCR

1. RNA extraction

For RNA extraction, an inoculum of an overnight bacterial culture was diluted 1:100 in LB Lennox and 1.5 mL of the culture was harvested at OD_{600} = 0.8. In the case of ethanol and hydrogen peroxide stress, the bacterial cells were harvested 20 minutes after addition of the stress agent to the culture.

RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, Canada). The bacterial cells were pelleted by centrifugation for 10 minutes at 5,000 x g and then frozen at -80°C for at least 60 minutes to aid cell lysis. The supernatant was decanted and 100 µL of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg/mL lysozyme (Qiagen, Mississauga, Canada) were added to the cell pellet. The contents were mixed and incubated at room temperature for 10 minutes. 350 µL of Buffer RLT (lysis buffer) containing 3.5 µL of β-mercaptoethanol were added to the mixture and mixed vigorously for ~ 10 seconds. 250 µL of 96% - 100% ice-cold ethanol (Sigma-Aldrich, Oakville, Canada) were added to the sample and mixed gently by pipetting and inverting. Upon a ~ 5 minute incubation with ethanol, the lysate (~ 700 µL) was transferred to a RNeasy Mini spin column placed in a collection tube (supplied with the kit). The lysate was centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. 700 µL of Buffer RW1 were added to the spin column and centrifuged for 15 seconds at 10,000 rpm. The spin column with the sample on the membrane was washed with 500 µL of Buffer RPE twice and the spin column was centrifuged for 15 seconds at 10,000 rpm after the first addition of Buffer RPE and for 2 minutes after the second addition of Buffer RPE as per manufacturer protocol. The RNeasy spin column was then placed in a new 1.5 mL collection

tube (supplied with the RNeasy Mini Kit, Qiagen) and the spin column membrane was washed with 30 µL of RNase-free water (supplied with the RNeasy Mini Kit, Qiagen) to elute mRNA by centrifugation for 1 minute at 10,000 rpm. Extracted mRNA was stored at -80°C.

2. cDNA synthesis

One microgram of the total RNA was used to synthesize the first-strand cDNA using a GoScript Reverse Transcriptase Kit (Promega, Madison, USA). Random primers (0.5 µg / reaction), experimental RNA (to a final quantity of 1 µg / reaction) and nuclease-free water were mixed to a final volume of 5 µL per reaction. The reactions were incubated at 70°C for 10 minutes and then cooled down on ice for at least 5 minutes. The reverse transcription reaction mixture was set up to a final volume of 15 µL and contained the GoScript™ 5x reaction buffer (4 µL), $MgCl_2$ to a final concentration of 2.5 mM per reaction, the dNTP mixture to a final concentration of 0.5 mM of each dNTP and the GoScript™ reverse transcriptase (1 µL). Nuclease-free water was added to the mixture to a final volume of 15 µL. The 15 µL aliquots of the reverse transcription reaction mixture were added to each 5 µL reaction containing first-strand cDNA (described above), combining, therefore, to a final volume of 20 µL.

A control negative-RT reaction was set up for every cDNA synthesis reaction by excluding the reverse transcriptase enzyme from the reaction mix to rule out genomic DNA contamination of cDNA samples.

3. Primer design, primer standardization and quantitative RT-PCR (qRT-PCR)

The transcription levels of three RND efflux pump genes, *adeB, adeG, adeJ*, and three outer membrane porin-encoding genes, *carO, oprD* and a gene encoding the 33kDa porin protein, were analyzed using qRT-PCR. The transcription levels of the response regulator genes of the selected

two-component systems were compared between *A. baumannii* ATCC 19606 type strain and two clinical isolates of *A. baumannii*, AB030 and AB031: *bfmR, VbIAciBau3967_0658, VbIAciBau3967_1097*, *VbIAciBau3967_2424*, *VbIAciBau3967_1128*, *VbIAciBau3967_1305*, *VbIAciBau3967_2809*, *VbIAciBau3967_3362*, *VbIAciBau3967_3515* and *VbIAciBau3967_1956*. The transcription levels of *adeR*, a response regulator of the AdeRS two-component system, were assessed under all tested environmental conditions.

All qRT-PCR primers used in the study were designed using Invitrogen OligoPerfect^{TM} Designer software available at www.invitrogen.com (Table 1). The primers were designed so that the expected length of the target amplicon was around 100 base pairs and melting temperature was approximately 60°C. The *hsp90* and *groEL* levels of transcription were used as a control for incubation temperature, salicylate exposure, ethanol and oxidative shock. The 16S rRNA-encoding gene was used as a housekeeping control for all environmental conditions tested.

Before the qRT-PCR reactions were set up, each primer set was standardized against gDNA of *A. baumannii* ATCC 19606 as a template to ensure the stringency and efficiency of the primers binding to their target sequences. The primers were standardized against up to five sets of ten-fold serial dilutions of *A. baumannii* ATCC 19606 genomic DNA (Appendix A). Primer standardization was considered a success if the binding efficiency (E-value) of a primer set was between 95%-105%, with the R^2-value of the slope of the line of the best fit of approximately 1.

The quantitative RT-PCR reactions were set up in triplicates for each of at least two biological replicates for every condition tested. The total volume of each reaction was 15 µL including 5 µL of the cDNA template (a 1 in 20 dilution in RNAse and DNAse free water of

cDNA obtained in the cDNA-synthesis reactions described above) with a final concentration of each primer at 300 nM. A "no reverse transcriptase" (NRT) control reaction was analyzed (with no reverse transcriptase enzyme added to the reaction) with the 16S rRNA primers only while a "no template" control (NTC) was set up for each primer set. The analysis of the results was carried out using the $2^{-\Delta\Delta Ct}$ method and CFX Manager 2.0 software (Biorad, Mississauga, Canada).

The $2^{-\Delta\Delta Ct}$ (Livak) method

The $2^{-\Delta\Delta Ct}$ method was used to quantify relative gene expression of the target genes relative to a chosen reference gene (16S rRNA-encoding gene and *fmcC*). The C_t values of the target genes (RND efflux pump and outer membrane protein-encoding genes as well as the genes encoding response regulators of the selected TCS) were normalized to those of the reference gene (16S rRNA-encoding gene) for both the test and control (calibrator) samples as per formula:

$$\Delta C_{t(test)} = C_{t(target,\ test)} - C_{t(reference,\ test)}$$

$$\Delta C_{t(calibrator)} = C_{t(target,\ calibrator)} - C_{t(reference,\ calibrator)}$$

The ΔC_t values of the test sample were subsequently normalized to the ΔC_t of the calibrator:

$$\Delta\Delta C_t = \Delta C_{t(test)} - \Delta C_{t(calibrator)}$$

The expression ratios of the target genes were calculated as follows:

$2^{-\Delta\Delta Ct}$ = Normalized expression ratio.

The obtained values for normalized expression ratio indicate fold change (increase or decrease) in the target gene expression relative to the calibrator (control) sample and were normalized to expression of the reference gene, 16S rRNA. The primer standardization curves with the E-values (efficiency of amplification) and the R^2-values of the lines of the best fit for all primer sets used in the study are shown in Appendix A.

Table 1. List of oligonucleotide primers used for qRT-PCR analysis

Target gene	Associated function	Primer name	Sequence (5'- 3')	Amplicon length, bp
adeB	RND efflux transporter	AdeB_R_RT	AATACTGCCGCCAATACCAG	106
		AdeB_F_RT	GGATTATGGCGACTGAAGGA	
adeJ	RND efflux transporter	AdeJ_F1_RT	CATCGGCTGAAACAGTTGAA	105
		AdeJ_R1_RT	GCCTGACCATTACCAGCACT	
adeG	RND efflux transporter	Ab3219_R_RT	GTGACTTGGGAAAGCCCATA	100
		Ab3219_F_RT	ACCGCTTTAGAGGTCGAACA	
33kDa	OM porin	Ab_33kD_F_RT	ATCCAAAACGACCAAGATGC	99
		Ab_33kD_R_RT	CAAAACCGATTGCCATGTTA	
oprD	OM porin	Ab_OprD_R_RT	AACAACGCCTACACCGAAAC	102
		Ab_OprD_F_RT	TTGGAGCAAAACCAAAACCT	
carO	OM porin	Ab_CarO_R_RT	TTGGAGCAAAACCAAAACCT	100
		Ab_CarO_F_RT	AGCAGTTCGTGGTCAAGAGG	
adeR	Response regulator	AdeR_F1_RT	GAGTGTTATTCGGGCCATGA	105
		AdeR_R1_RT	CCAACCGTTTAATTCGGGTA	
fmcC	Fumarase C	FmcC_R_RT	CATGAATAGCCGTTGGGAAT	100
		FmcC_F_RT	AGCACAAAAGCCGGTACATC	
16S	Ribosomal RNA	Ab_16S_RT_Ra	CGTAAGGGCCATGATGACTT	104
		Ab_16S_RT_Fa	ACATCTCACGACACGAGCTG	
hsp90	Heat-shock protein	Hsp90_0294_RT_R	TCAGCATCTTGCAAGTCACC	100
		Hsp90_0294_RT_F	GACGAGTGGGCAATGAACTT	
groEL	Heat-shock protein	GroEL_2664_RT_F	AAGAAAGACCGCGTAGACGA	102
		GroEL_2664_RT_R	CATTTACAGCGCGAACAAGA	
VbIAciBau3967_1956	Helix-turn-helix, Fis-type	A1S_0236_RT_R	AACGACGGGTTGTTTCTACG	100
		A1S_0236_RT_F	TATCGTTCGCCAACAACATC	106
VbIAciBau3967_3515	Transcriptional regulator	A1S_1232_RT_R	TCCCCTTTAGGGGTTAGCAC	104
		A1S_1232_RT_F	TCTGACCGGCTGAAAAAGTT	103
VbIAciBau3967_3362	Sensor protein, PhoR	A1S_3375_RT_R	TGCAAGCAGACGGTATTCTG	105
		A1S_3375_RT_F	CAATTGATGCCAATGGTTTG	105
VbIAciBau3967_2809	Sensory histidine	A1S_2751_RT_R	ATACGGGCAAGCAACTCATC	102

	kinase	A1S_2751_RT_F	TTACAAAACCGTGTCGATGG	98
VbIAciBau3967_1305	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	A1S_0748_RT_R	ACCGTTCAACGTTACCGAAC	105
		A1S_0748_RT_F	CGCCGTACGGATAAAACTGT	104
VbIAciBau3967_1128	Nitrogen regulation protein	A1S_1978_RT_F	CGCCCGATGTCATCTTAACT	103
		A1S_1978_RT_R	GTCGGGGTGAGAGTTTTTCA	102
VbIAciBau3967_2424	Osmolarity sensory His kinase	A1S_3229_RT_F	CTCGTGAGGGTCAAATCGTT	100
		A1S_3229_RT_R	TGTCGCGTGTTAAAGGTTCA	98
VbIAciBau3967_1097	Response regulator	A1S_2006_RT_F	GTTGAAAAGGCCAAAGTGCT	102
		A1S_2006_RT_R	GTGCCATTTCTCCAATCGTT	98
VbIAciBau3967_0222	Osmosensitive K^+ channel His kinase	A1S_1753_RT_F	CATGAATGGAAAGCAAGCAA	106
		A1S_1753_RT_R	CCAACCGTTTAATTCGGGTA	105
VbIAciBau3967_0658	Two-component hybrid sensor and regulator	A1S_1753_RT_R	TTCACTGTCCGTGGACTCAG	105
		A1S_1394_RT_F	AAATATTGCCCGCAGTTGAC	103
bfmR	Response regulator	BfmR_RT_R	CATGAGATACCGCCCTCATT	101
		BfmR_RT_F	TCCTATTGAGGGAAGCGATG	100

D SUSCEPTIBILITY ASSAY

Susceptibility assays were performed using the disc diffusion method (Bauer *et al*., 1966) with the following conditions tested: 30°C, 37°C and 42°C incubation temperatures, 4 mM salicylate and iron(III)-abundant conditions (100 μM Fe^{3+}). LB Lennox and Mueller-Hinton agar plates were supplemented with the aforementioned chemical agents at the specified concentrations and incubated at appropriate temperature for 18 hours in a stationary incubator. The susceptibility assay values for *A. baumannii* ATCC 19606 under the specified environmental conditions were determined for the following antimicrobials: imipenem, ciprofloxacin, gentamycin, clindamycin, ceftriaxone and trimethoprim/sulfamethoxazole combination.

E STATISTICAL ANALYSIS OF THE DATA

For all significance analysis of the data an unpaired Student's T-test with the 95% confidence interval was performed using MS Excel (Microsoft). Plotted normalized fold expression values for each target gene represent an average normalized fold expression of 3 technical replicates. The error bars represent the standard deviation of the normalized fold expression values of the target genes of 3 technical replicates for each gene and condition.

3. RESULTS

A. EXPRESSION OF RND EFFLUX PUMPS AND PORIN PROTEINS

1. Effect of incubation temperature on RND efflux pumps and porin protein expression

The transcription levels of the *adeB, adeJ, adeG* genes encoding the inner membrane transporters of the AdeABC, AdeIJK and AdeFGH RND efflux pumps respectively, and the *33kDa, carO* and *oprD* genes encoding outer membrane porins in *Acinetobacter baumannii* ATCC 19606 strain were compared at 30°C, 37°C and 42°C.

The operons encoding the AdeABC and AdeFGH RND efflux pumps (the *adeB* and *adeG* genes, respectively) were the most temperature-sensitive among the efflux systems analyzed. The data suggests that *adeB* is ~5-fold downregulated at 30°C relative to 37°C. However, no statistically significant difference between the transcription levels of the *adeB* gene at 42°C relative to 37°C was detected (Fig. 1). The transcription levels of the *adeJ* gene were reduced ~1.2-fold and ~1.4-fold at 30°C and 42°C, respectively. The *adeG* gene was found to be ~2.8 fold upregulated in response to 42°C. The *adeR* gene, the response regulator of the AdeRS two-component system that controls expression of the *adeABC* operon, was found to be ~3-fold and ~2-fold upregulated in response to 30°C and 42°C, respectively, relative to 37°C (Fig. 2).

All three OM porins genes analyzed in the study, *33kDa, carO* and *oprD*, were downregulated at 30°C and 42°C in comparison to 37°C (Fig. 1). The 33kDa OM porin-encoding gene was found to be the most sensitive of the three OM porin genes tested showing a ~4-fold downregulation at 42°C relative to 37°C but only a 1.2-fold downregulation at 30°C (Fig. 1). Remarkably, the transcription levels of the 33kDa porin-encoding gene were found to be ~3.2-fold lower at 42°C when compared to its transcription levels at 30°C.

The other two OM porin-encoding genes, *carO* and *oprD*, were ~1.64-1.8-fold downregulated at 30°C and 42°C relative to 37°C. No statistically significant difference in the expression levels of either of the *carO* or *oprD* genes between 30°C and 42°C was detected (Fig. 1).

To confirm that *A. baumannii* was sensing the temperature change, we have also evaluated the transcriptional response of the *hsp90* gene encoding a heat-shock response protein. *hsp90* was ~3.3-fold downregulated when exposed to 30°C relative to its expression levels at 37°C. In contrast, *hsp90* was ~2-fold upregulated in response to 42°C in comparison to 37°C control conditions (Fig. 3).

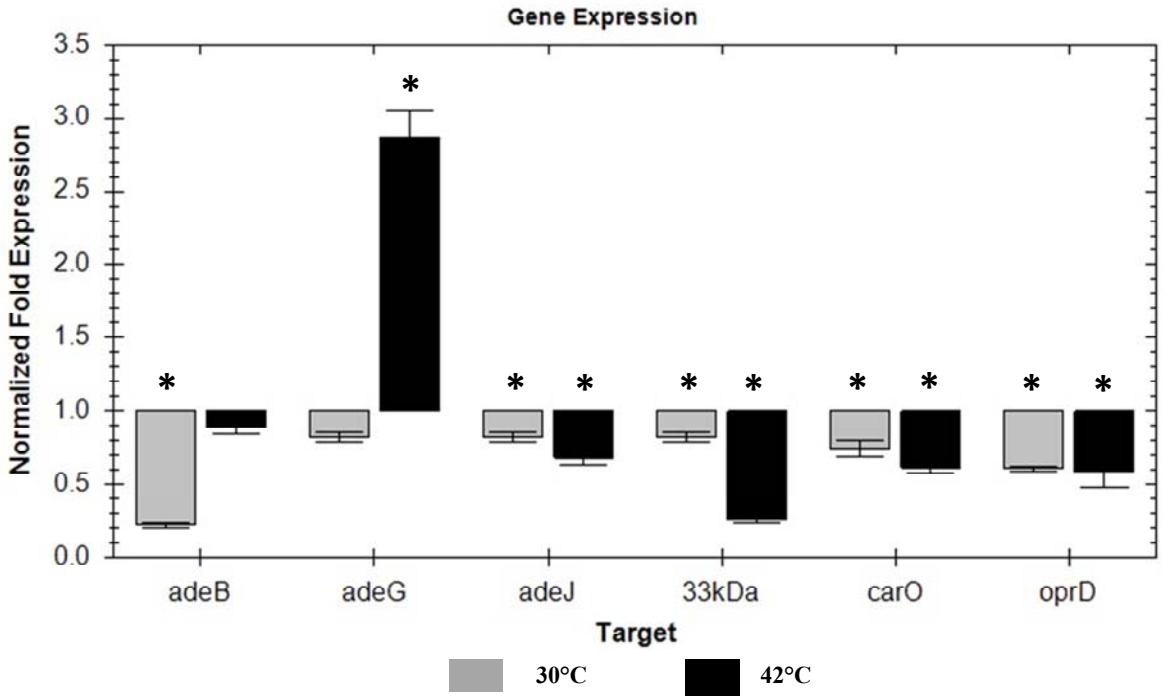

Figure 1. Comparative expression of the inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and the outer membrane porin-encoding genes, *33kDa, carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to 30°C and 42°C incubation temperatures (relative to 37°C). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to 37°C as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

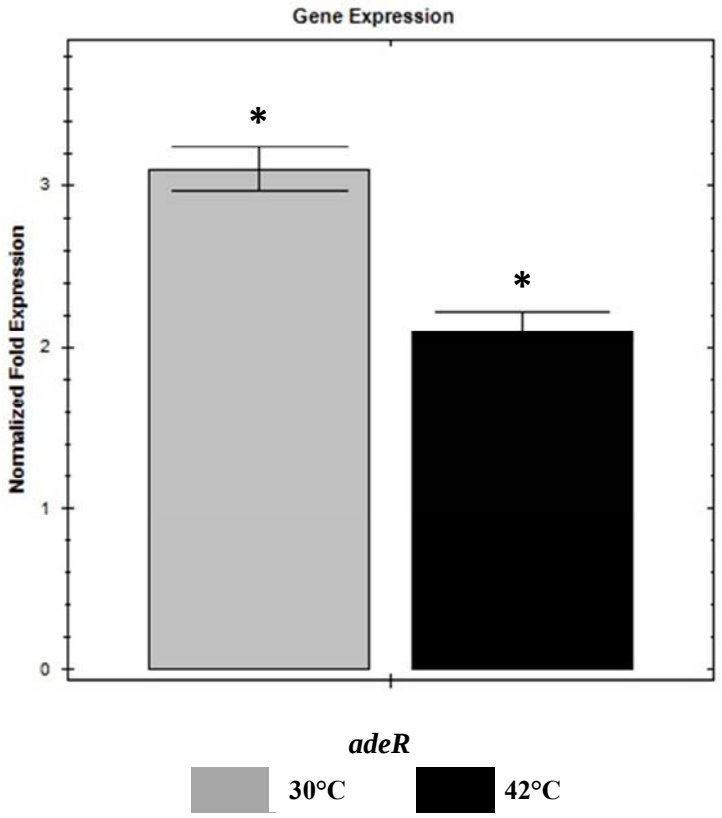

Figure 2. Comparative expression of the *adeR* gene in *A. baumannii* ATCC 19606 upon exposure to 30°C and 42°C incubation temperatures (relative to 37°C). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to 37°C as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

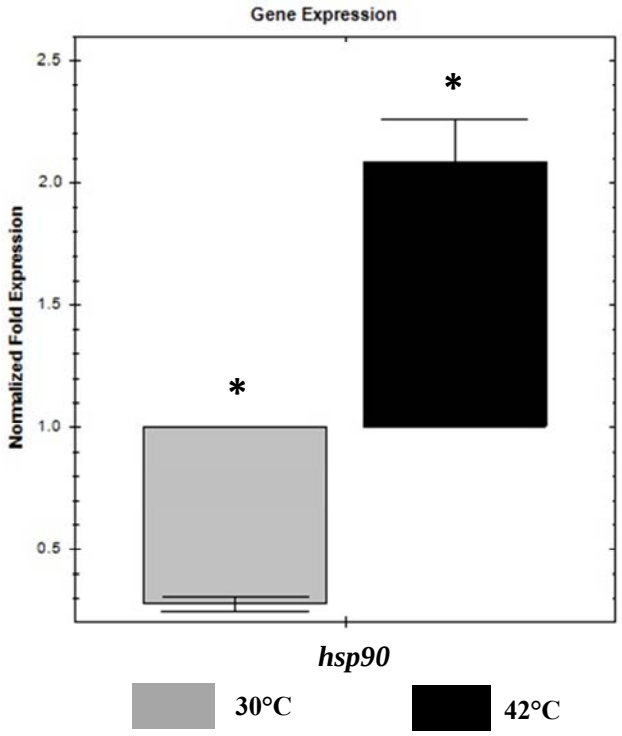

Figure 3. Comparative expression of the *hsp90* heat-shock-response gene in *A. baumannii* ATCC 19606 upon exposure to 30°C and 42°C incubation temperatures (relative to 37°C). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to 37°C as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

2. Effect of iron(III) - depleted/repleted conditions on expression of RND efflux pumps and outer membrane porin proteins

The transcription levels of the *adeB, adeG* and *adeJ* genes and the genes encoding 33kDa, CarO and OprD porins were analyzed under iron(III)-depleted (150 μM ferrichrome siderophore added to the media) and iron(III)-repleted conditions (100 μM Fe^{3+} supplied into the media). Consistent downregulation of all three RND efflux pumps under iron(III)-rich conditions was observed: ~ 4-fold for *adeB* and ~ 2-fold for both *adeG* and *adeJ* genes (Fig. 4). *adeB* was the only gene amongst the ones encoding RND inner membrane transporters to be upregulated, ~1.4-fold, in response to iron(III)–depleted conditions (Fig. 4). The transcription levels of the *adeR* gene were also quantified. Interestingly, it was found that the *adeR* gene expression was ~2.2-fold upregulated in response to iron(III)-depleted conditions relative to control (Fig. 5).

Amongst the outer membrane porins, the CarO-encoding gene was the only one to be upregulated: ~1.9-fold under iron(III)-rich conditions and ~1.6-fold under iron(III)-depleted conditions (Fig. 4). In contrast, *33kDa* was downregulated in response to both iron(III)-rich and iron(III)-depleted conditions, ~2-fold and ~1.5-fold, respectively. *oprD* was found to be ~2.5-fold downregulated in response to iron(III)-rich conditions. No statistically significant difference in the transcription levels of *oprD* in response to iron(III)-depleted conditions was observed (Fig. 4).

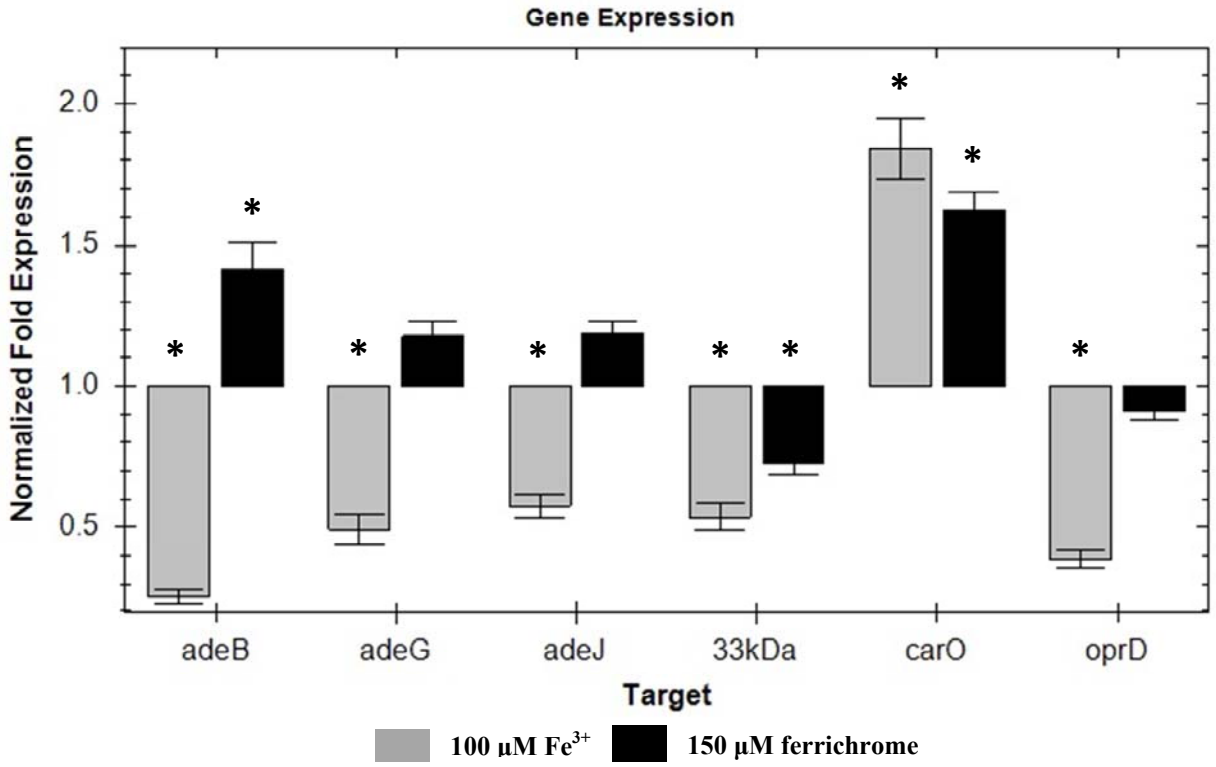

Figure 4. Comparative expression of the inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and three outer membrane porin-encoding genes, *33kDa, carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to iron(III)-depleted (150 µM ferrichrome) and iron(III)-abundant (100 µM Fe^{3+}) conditions (relative to control with no ferrichrome or iron(III) added to the media). The gene expression data is representative of three biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control (no ferrichrome or iron(III) added to the media) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

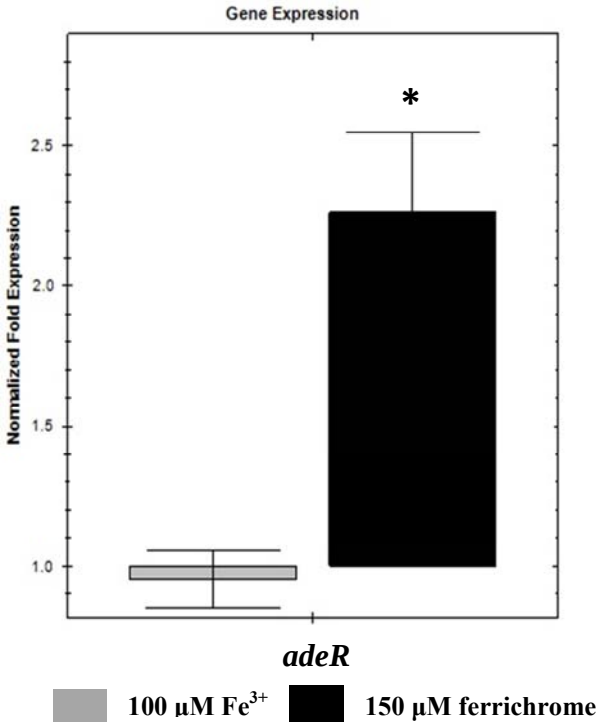

Figure 5. Comparative expression of the *adeR* gene in *A. baumannii* ATCC 19606 upon exposure to iron (III)-depleted (150 μM ferrichrome) and iron(III)-abundant (100 μM Fe^{3+}) conditions (relative to control with no ferrichrome or iron(III) added to the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control (no ferrichrome or iron(III) added to the media) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

3. Effect of ethanol shock on transcription of RND efflux pumps, porins and heat stress response genes

The *adeG* and *adeJ* genes responded with a ~2-fold downregulation to a 0.5% v/v ethanol treatment (Fig. 6). The *adeB* and *adeG* genes were found to be ~1.5-fold downregulated in response to 0.5% v/v and 1.1% v/v ethanol shock, respectively. The *adeR* gene showed a ~2.7-fold upregulation in response to both 0.5% and 1.1% v/v ethanol shock (Fig. 7).

Amongst the porin genes, no statistically significant change was observed in the transcription levels of the *33kDa* and *oprD* genes under either of the ethanol concentrations when compared to control (no exogenous ethanol supplied into the media). In contrast, *carO* responded with a ~1.9-fold upregulation in response to 1.1% v/v ethanol and a slight downregulation, ~1.3-fold, in response to 0.5% v/v ethanol (Fig. 6). The *hsp90* gene was found to be ~1.5-fold upregulated in response to 1.1% v/v ethanol shock but not to 0.5% v/v ethanol (Fig. 8).

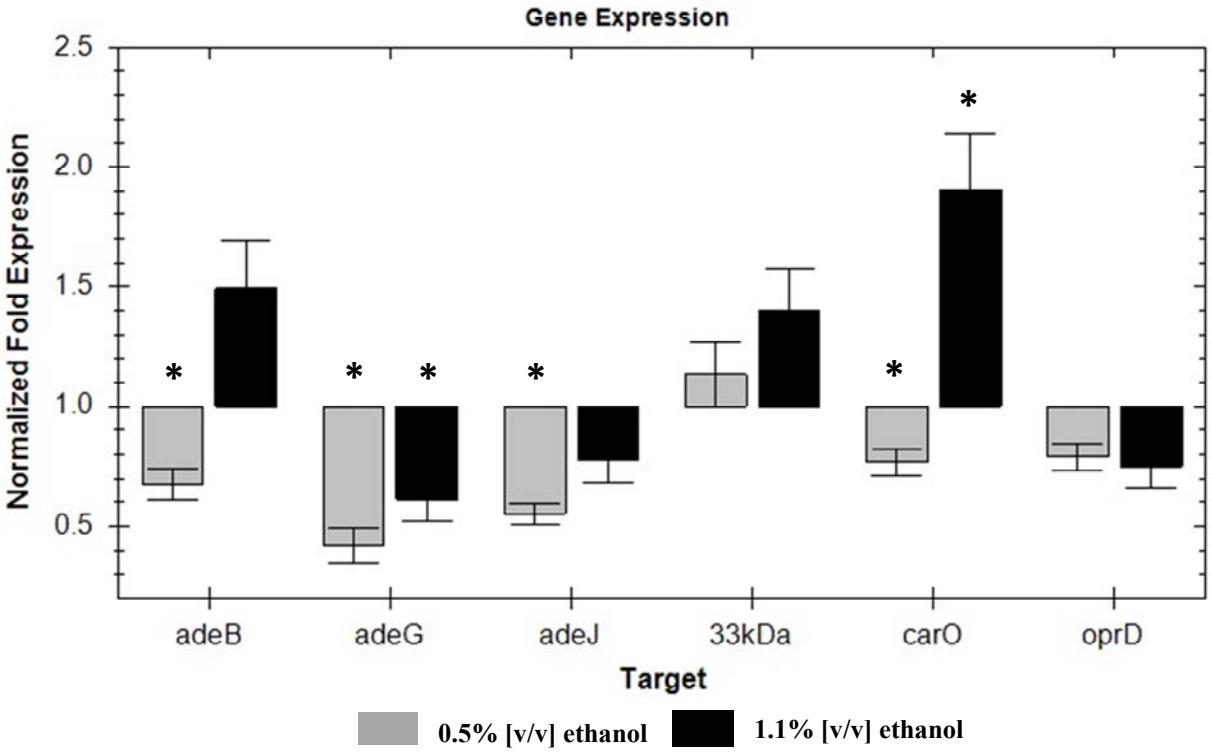

Figure 6. Comparative expression of the inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and three outer membrane porin-encoding genes, *33kDa, carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to 0.5% and 1.1% v/v ethanol (relative to control with no ethanol supplied into the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control (no ethanol added to the media) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

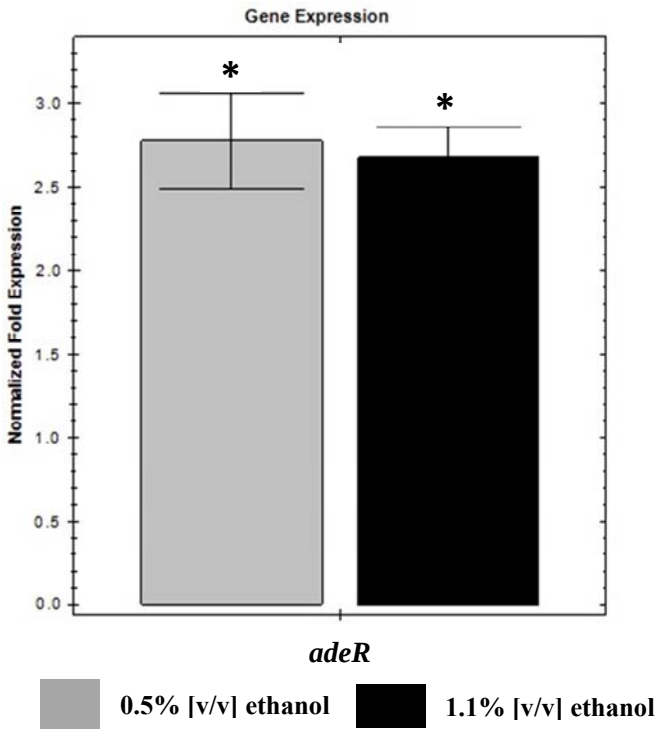

Figure 7. Comparative expression of the *adeR* gene in *A. baumannii* ATCC 19606 upon exposure to 0.5% and 1.1% v/v ethanol shock treatment (relative to control with no ethanol added to the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

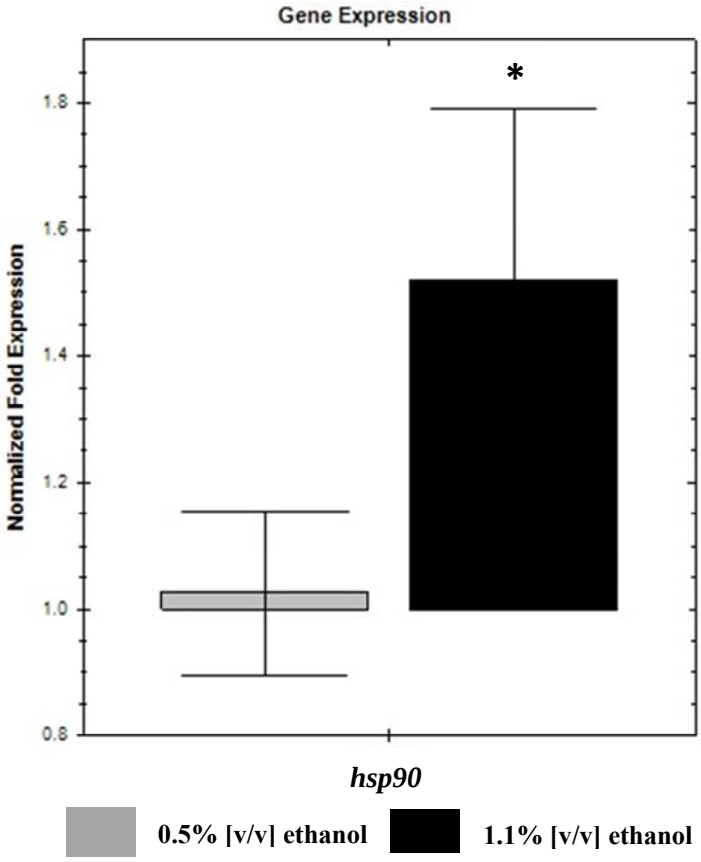

Figure 8. Comparative expression of the *hsp90* heat-shock-response gene in *A. baumannii* ATCC 19606 upon exposure to 0.5% and 1.1% v/v ethanol shock treatment (relative to control with no ethanol added to the media).The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control (no ethanol added to the medium) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

4. Effect of oxidative stress on transcription of RND efflux pumps and porins

Amongst the RND efflux genes, both *adeB* and *adeG* genes were over 2-fold upregulated in response to both 1 mM and 2 mM hydrogen peroxide, with *adeB* showing almost a ~2.6-fold upregulation upon exposure to 2 mM hydrogen peroxide (Fig. 9). The levels of transcription of the *adeR* gene were ~2.5-fold and ~2.8-fold elevated upon exposure to 1 mM and 2 mM hydrogen peroxide in comparison to control (Fig. 10). The *adeJ* gene responded to oxidative stress with a ~1.8-fold and a ~1.5-fold upregulation in response to 1 mM and 2 mM hydrogen peroxide, respectively.

Amongst porins, the OprD-encoding gene was the only one upregulated in response to oxidative stress, showing a ~1.8-fold and a ~3.5-fold upregulation in response to 1 mM and 2 mM hydrogen peroxide treatment, respectively (Fig. 9). The CarO-encoding gene showed a ~1.6-fold downregulation upon exposure to 2 mM hydrogen peroxide but no significant change in its transcription levels was observed in response to 1 mM hydrogen peroxide (Fig. 9). In contrast, the 33kDa porin-encoding gene showed a ~2-fold downregulation upon exposure to 1 mM hydrogen peroxide and no statistically significant change in its transcription levels in response to 2 mM hydrogen peroxide (Fig. 9).

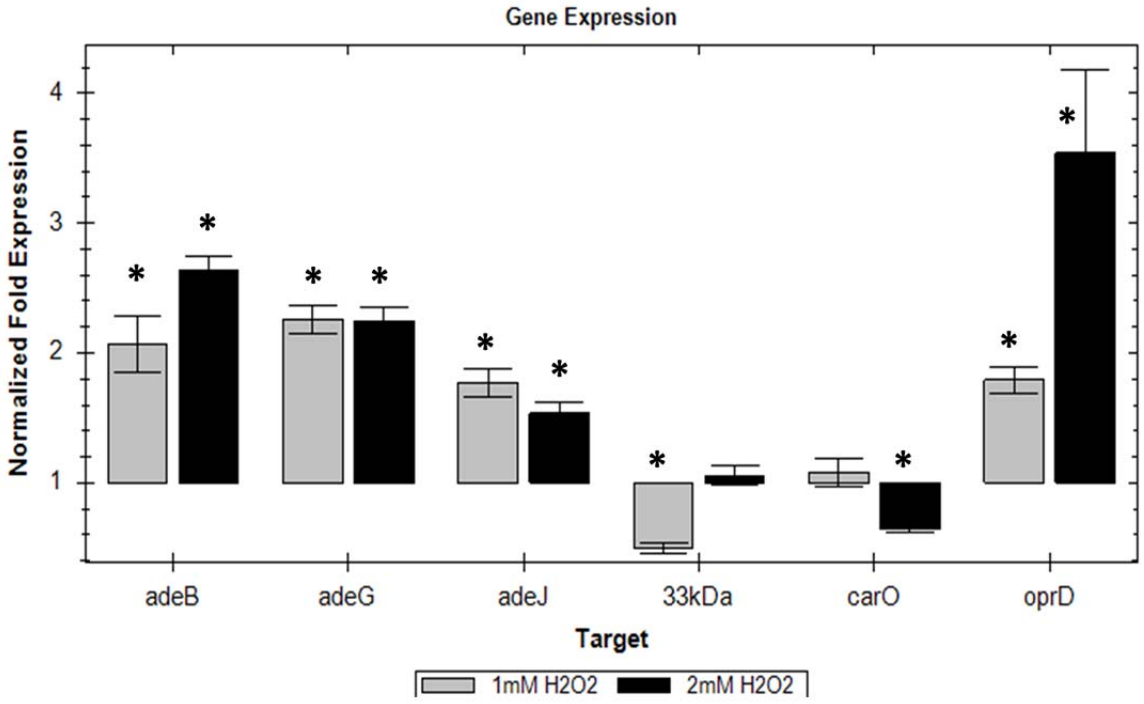

Figure 9. Comparative expression of three inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and three outer membrane porin-encoding genes, *33kDa, carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to 1 mM and 2 mM H_2O_2 (relative to control with no hydrogen peroxide supplied into the media). The gene expression data is representative of three biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control (no hydrogen peroxide added to the media) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

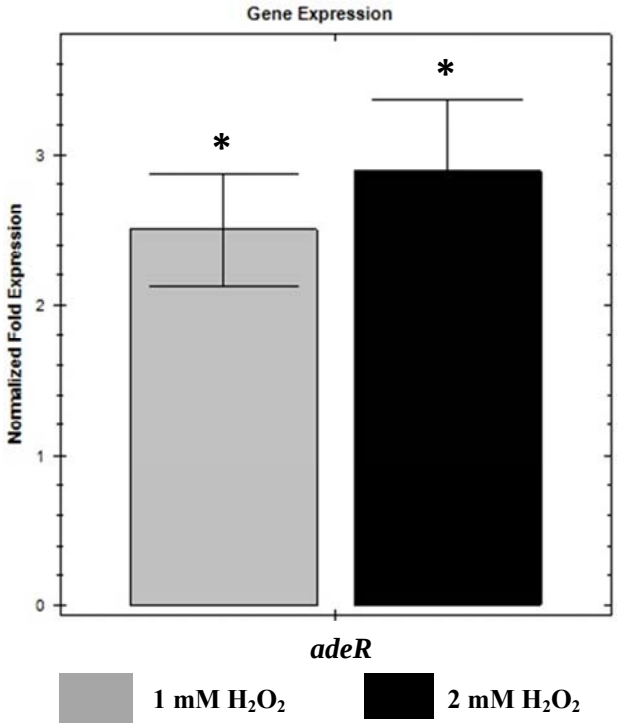

Figure 10. Comparative expression of the *adeR* gene in *A. baumannii* ATCC 19606 upon exposure to 1 mM and 2 mM H_2O_2 (relative to control with no hydrogen peroxide added to the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

5. Effect of salicylate exposure on expression of RND efflux pumps and outer membrane porins

The *adeB* gene was the most sensitive to the presence of salicylate in the growth media: a ~1.6-fold downregulation was observed upon exposure of *A. baumannii* ATCC 19606 to 2.5 mM sodium salicylate and a ~2.5-fold downregulation upon exposure to 4 mM salicylate (Fig. 11). The transcription levels of the *adeR* gene were elevated ~2.7-fold in response to 2.5 mM salicylate but were reduced ~1.3-fold in response to 4 mM salicylate (Fig. 12). The change in the transcription levels of the *adeG* and *adeJ* genes was not found to be statistically significant for either of the two concentrations of salicylate (Fig. 11).

Of the three outer membrane porins tested, the OprD-encoding gene expression was the only one sensitive to a salicylate exposure: a ~2.3-fold and a ~1.4-fold downregulation of *oprD* were observed in response to 2.5 mM and 4 mM salicylate treatment, respectively. The 33-kDa porin-encoding gene and *carO* were not found to be significantly affected by salicylate in the growth media (Fig. 11).

It was also found that the *groEL* gene expression was significantly downregulated upon exposure of *A. baumannii* ATCC 19606 to salicylate: ~4-fold and over 5-fold in response to 2.5 mM and 4mM sodium salicylate, respectively (Fig. 13).

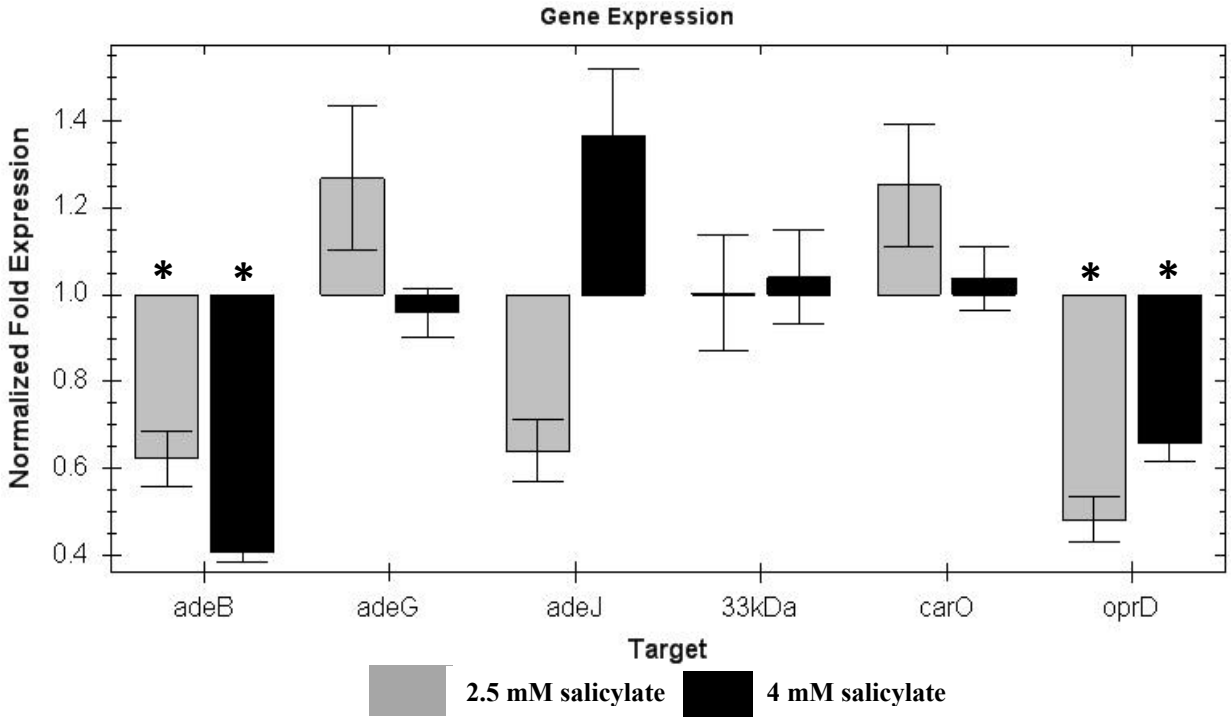

Figure 11. Comparative expression of the inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and three outer membrane porin-encoding genes, *33kDa, carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to 2.5 mM and 4 mM sodium salicylate (relative to control with no sodium salicylate supplied into the media). The gene expression data is representative of three biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

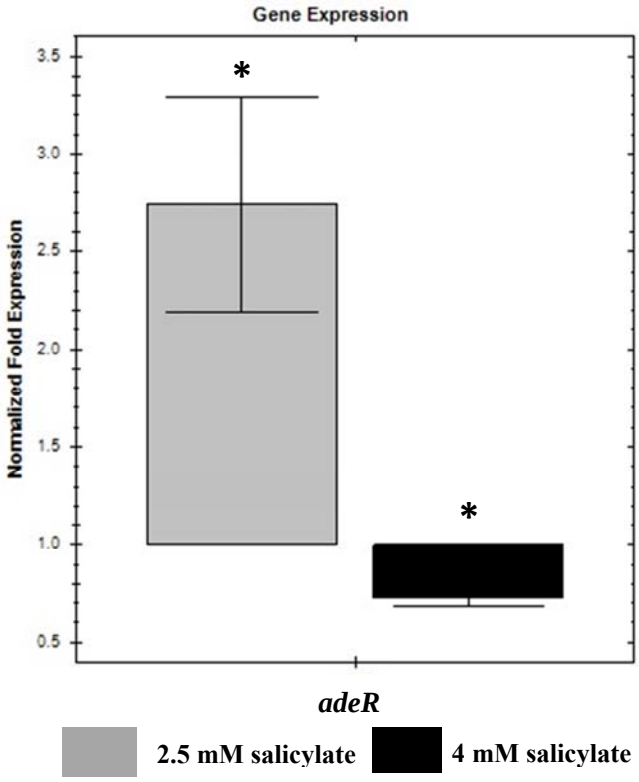

Figure 12. Comparative expression of the *adeR* gene in *A. baumannii* ATCC 19606 upon exposure to 2.5 mM and 4 mM sodium salicylate (relative to control with no sodium salicylate added to the media). The gene expression data is representative of three biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

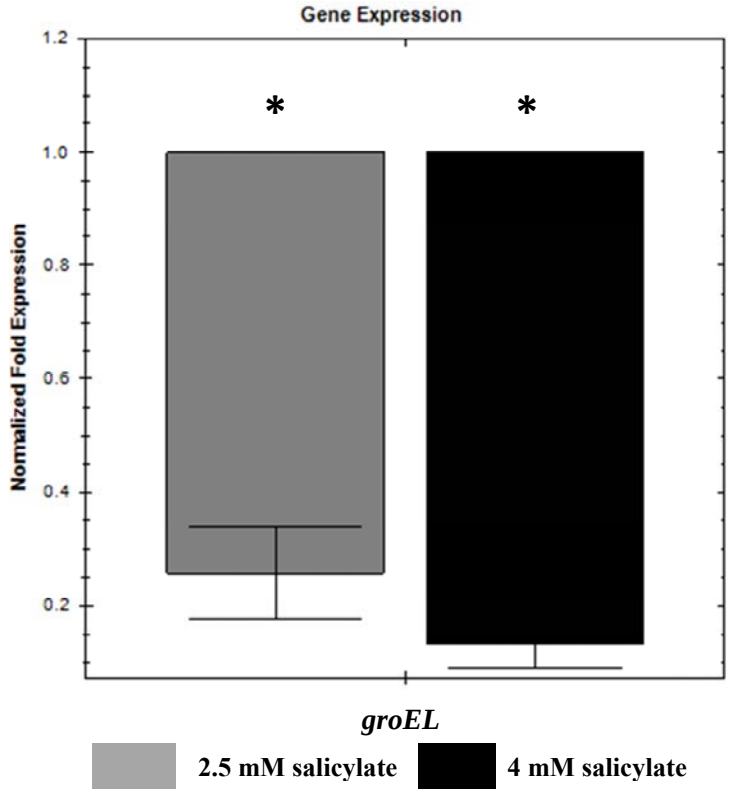

Figure 13. Expression of the *groEL* heat-shock-response gene in *A. baumannii* ATCC 19606 upon exposure to 2.5 mM and 4 mM sodium salicylate (relative to control with no salicylate added to the media).The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control (no salicylate added to the media) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

6. Effect of steady-state osmotic stress on expression of RND efflux pumps and outer membrane proteins

Steady-state osmotic stress conditions were achieved by adding 0.2 M and 0.3 M sucrose to the growth media throughout the duration of the experiment until the *A. baumannii* culture was harvested for mRNA extraction. Of the RND efflux pump-encoding genes, the *adeB* gene was the most sensitive to osmotic stress showing a ~2.5-fold downregulation in response to 0.3 M sucrose (Fig. 14). The changes in expression of the *adeG* and *adeJ* genes were not found to be statistically significant.

The outer membrane porin-encoding genes were found to be more responsive, on average, to steady-state osmotic stress than the genes encoding RND efflux pumps. The 33-kDa porin-encoding gene expression was ~2-fold and over 3-fold downregulated in response to 0.2 M and 0.3 M sucrose, respectively (Fig. 14). The CarO-encoding gene expression was ~1.25-fold downregulated in response to 0.3 M sucrose when compared to control conditions – a very small change in expression (although shown to be statistically significant) that is unlikely to have any physiological impact on the bacterial cells. The OprD-encoding gene expression was found to be over 2.5-fold and ~2-fold downregulated in response to 0.2 M and 0.3 M sucrose, respectively (Fig. 14). Surprisingly, it was found that the *adeR* gene, involved in the control of the expression of the AdeABC RND efflux pump, was upregulated ~6.5-fold in response to 0.2 M sucrose in the media. In contrast, a change in the transcription levels of the *adeR* gene in response to 0.3 M sucrose was not found to be statistically significant (Fig. 15). This pattern of the *adeR* expression, therefore, contradicts the pattern of expression of the *adeB* gene in response to the same osmotic stress conditions.

It was also found that the *groEL* gene was downregulated in response to steady-state osmotic shock. The gene was found to be 5-fold and 10-fold downregulated in response to 0.2 M and 0.3 M sucrose, respectively (Fig. 16).

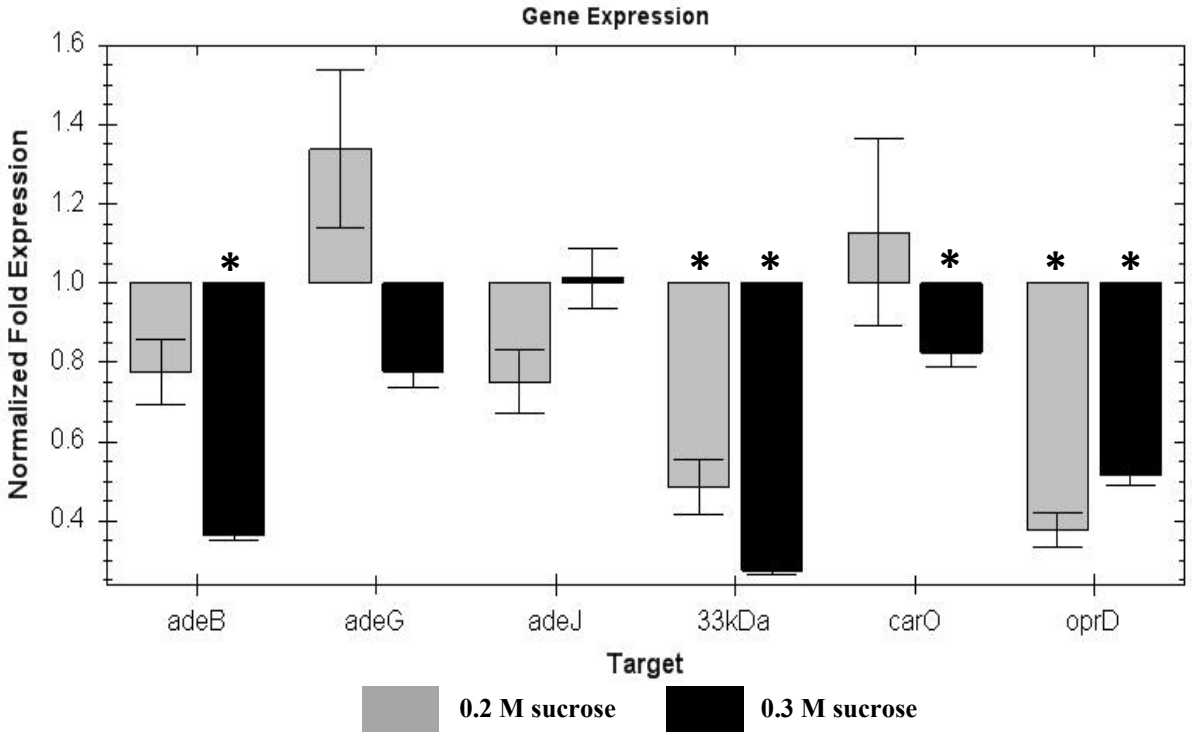

Figure 14. Comparative expression of the inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and three outer membrane porin-encoding genes, *33kDa, carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to 0.2 M and 0.3 M sucrose (relative to control with no sucrose supplied into the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

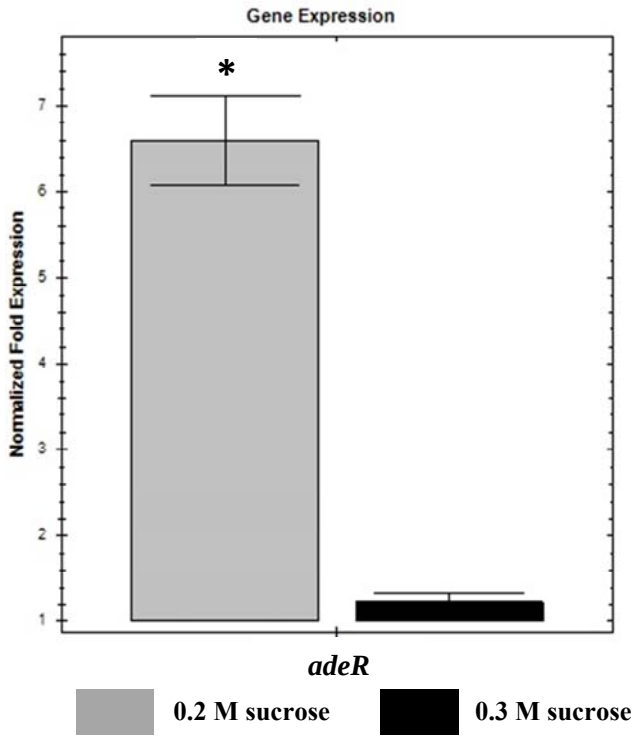

Figure 15. Comparative expression of the *adeR* gene in *A. baumannii* ATCC 19606 upon exposure to 0.2 M and 0.3 M sucrose (relative to control with no sucrose added to the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

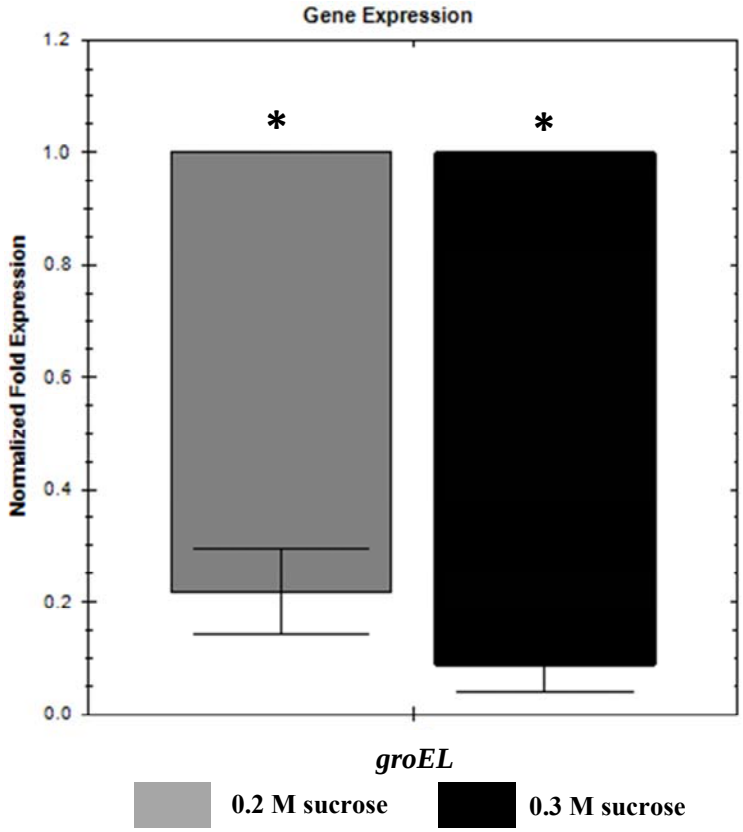

Figure 16. Expression of the *groEL* heat-shock-response gene in *A. baumannii* ATCC 19606 upon exposure to 0.2 M and 0.3 M sucrose (relative to control with no sucrose added to the media).The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target gene relative to control (no sucrose added to the medium) as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

7. Effect of blue light on expression of RND efflux pumps and outer membrane porins

The transcription levels of the RND efflux pumps and three outer membrane porins in *A. baumannii* ATCC 19606 were assessed in response to a blue-light exposure and compared to their expression levels under dark conditions. The only statistically significant difference in the expression levels in response to blue light was observed for the *adeJ* gene, which showed a ~1.3-fold downregulation relative to its expression under dark conditions (Fig. 17). The rest of the genes encoding the RND efflux inner membrane transporters and outer membrane porins did not respond to a blue light exposure.

8. Susceptibility assay of *A. baumannii* ATCC 19606 in response to environmental conditions

A change in incubation temperature proved to be the most potent environmental factor capable of altering the susceptibility of *A. baumannii* ATCC 19606 towards different antimicrobials. Incubation of the bacteria at 42°C resulted in a decrease of *A. baumannii* susceptibility towards imipenem, gentamicin and ceftriaxone reflected in the increase of the diameters of the zones of clearing: from 32 mm to 34 mm for imipenem, from 10.5 mm to 12 mm for gentamicin and from 12.5 mm to 15 mm for ceftriaxone (Table 2). A significant increase in *A. baumannii* susceptibility towards imipenem (an increase of the zone of clearing from 32 mm to 34.5 mm) and ciprofloxacin (an increase of the zone of clearing from 21.5 mm to 24 mm) was also observed at 30°C incubation temperature (relative to 37°C).

In response to the 4 mM salicylate exposure, the susceptibility of *A. baumannii* ATCC 19606 to imipenem increased (an increase of the zone of clearing from 32 mm to 34.5 mm) but its susceptibility to ciprofloxacin decreased as reflected in a decreased zone of clearing, from

21.5 mm to 19.5 mm (Table 2). A slight decrease of *A. baumannii* susceptibility to ciprofloxacin has been also observed in response to iron(III)-rich conditions as reflected in the reduction of the zone of clearing from 21.5 mm to 20.5 mm.

Lastly, exposure of *A. baumannii* ATCC 19606 to 1.1% v/v ethanol shock resulted in the organism's increase of susceptibility towards imipenem and ceftriaxone: the diameters of the zones of clearing increased from 32 mm to 33.5 mm and from 12.5 mm to 14 mm, respectively (Table 2). A slight decrease of susceptibility in response to ethanol shock was observed towards gentamicin as the diameter of the zone of clearing was reduced from 10.5 mm to 9.5 mm (Table 2).

A. baumannii ATCC 19606 was shown to be resistant to trimethoprim/sulfamethoxazole antibiotic cocktail and clindamycin. It was shown to be the least sensitive to gentamycin (a clearing zone of 10.5 mm for control conditions) and the most sensitive to imipenem (a clearing zone of 32 mm in diameter for control conditions) (Table 2).

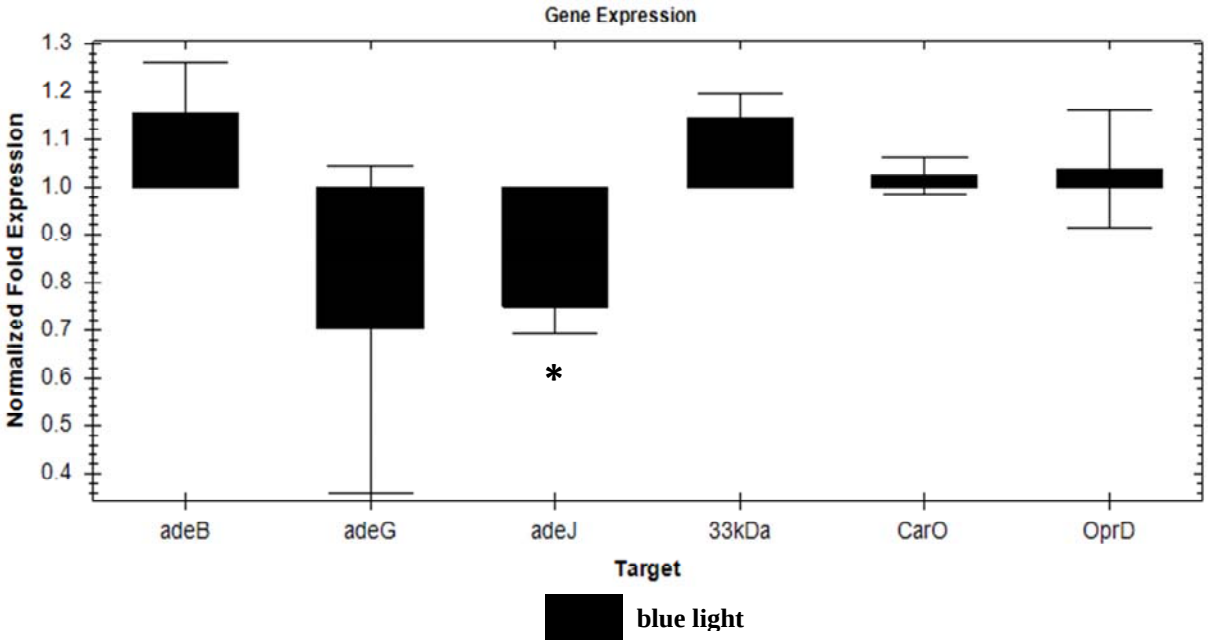

Figure 17. Comparative expression of the inner membrane transporter-encoding genes, *adeB, adeG* and *adeJ*, and three outer membrane porin-encoding genes, *33kDa*, *carO* and *oprD*, in *A. baumannii* ATCC 19606 upon exposure to blue light (relative to control where bacterial culture was grown while exposed to the dark conditions). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

Table 2. Susceptibility of *A. baumannii* ATCC 19606 strain in response to selected antibiotics upon exposure to different environmental stress conditions. Values represent the diameters of the clearing zones (in millimeters) calculated as an average of three technical replicates for each antibiotic and each condition tested. The data is representative of three biological replicates.

Antibiotic/ Condition	Resistance associated protein(s)	30°C	37°C	42°C	[1]Sal	[2]Fe^{3+}	[3]EtOH
Imipenem	OprD, 33 kDa, CarO	34.5	32	34	34.5	31.5	33.5
Ciprofloxacin	AdeABC, AdeFGH, AdeIJK	24	21.5	22	19.5	20.5	21.5
Gentamicin	AdeABC	11.5	10.5	12	10	10.5	9.5
Trimethoprim-Sulfamethoxazole	AdeABC, AdeFGH, AdeIJK	0	0	0	0	0	0
Clindamycin	AdeFGH	0	0	0	0	0	0
Ceftriaxone	AdeABC	12.5	12.5	15	12.5	12.5	14

[1]Sodium salicylate (4 mM); [2]Ferric chloride (100 μM); [3]Ethanol (1.1% [vol/vol])

B EXPRESSION OF TWO-COMPONENT SYSTEMS

1. Comparative expression of two-component systems in *A. baumannii* ATCC 19606 type strain and *A. baumannii* clinical isolates, AB030 and AB031

The transcription levels of ten response regulator genes were assessed in a type strain of *A. baumannii*, ATCC 19606, and compared to their transcription levels in the two clinical isolates of *A. baumannii*, strains AB030 and AB031. The latter two clinical isolates were selected due to strain's ability to form biofilms (strain AB031) and a multi-drug resistant phenotype (strain AB030). From ten response regulator genes analyzed in this study, six of them - *VbIAciBau3967_1305*, *VbIAciBau3967_1128, VbIAciBau3967_3362, VbIAciBau3967_0658* and *bfmR* were significantly downregulated in both *A. baumannii* clinical isolates in comparison to ATCC 19606 type strain (Fig. 18, 19). *VbIAciBau3967_1305, VbIAciBau3967_3362*, *VbIAciBau3967_0658* and *bfmR* genes showed a ~2.5-fold downregulation in both clinical isolates. Slightly higher levels of downregulation were observed for *VbIAciBau3967_3515* in AB030 and AB031. It showed a ~5-fold downregulation in AB030 and a ~7-fold downregulation in AB031 in comparison to *A. baumannii* ATCC 19606 type strain.

The *VbIAciBau3967_1128* gene was downregulated ~2.5 and ~1.5-fold in strains AB030 and AB031, respectively (Fig. 18). Remarkably, the *VbIAciBau3967_1128* gene was the only response regulator which levels of expression were found to be greater, approximately 2-fold, in AB031 in comparison to AB030 (Fig. 18). Only one of the response regulators genes analyzed, *VbIAciBau3967_1097*, was upregulated, over 4-fold, in AB030 in comparison to *A. baumannii* ATCC 19606 strain (Fig. 18).

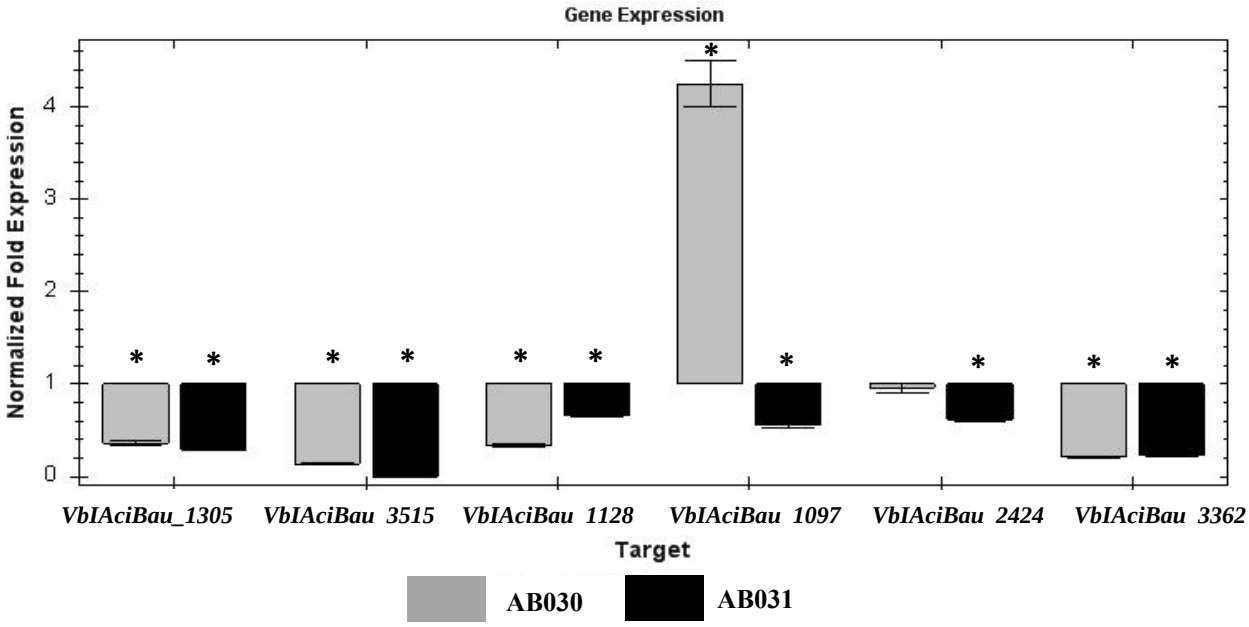

Figure 18. Comparative expression of the response regulators genes of the selected two-component systems in two clinical isolates of *A. baumannii*, strains AB030 and AB031. The expression levels are shown relative to the expression levels of the response regulator genes in *A. baumannii* ATCC 19606 type strain. The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to ATCC 19606 strain as determined by an unpaired Student's *t* test (at 95% confidence interval). The error bars represent standard deviation of the normalized fold expression values.

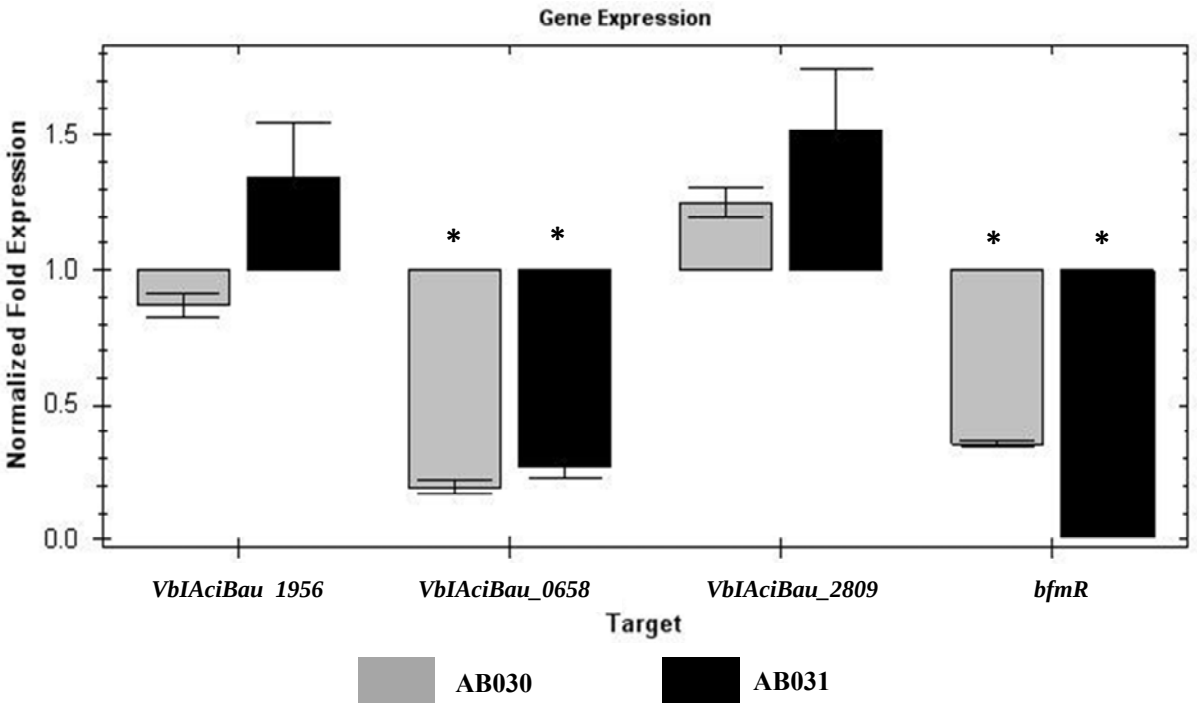

Figure 19. Comparative expression of the response regulators genes of the selected two-component systems in two clinical isolates of *A. baumannii*, strains AB030 and AB031. The expression levels are shown relative to the expression levels of the same response regulator genes in *A. baumannii* ATCC 19606 type strain. The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to *A. baumannii* ATCC 19606 type strain as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

2. Comparative expression of two-component systems under iron(III)-depleted and iron(III)-repleted conditions

The transcription levels of 7 response regulators genes were assessed for their response to iron(III) levels in the growth media. Six genes, *VbIAciBau3967_1305, VbIAciBau3967_3515, VbIAciBau3967_0222, VbIAciBau3967_3362, VbIAciBau3967_1097* and *bfmR* were differentially expressed in response to iron(III) levels (Fig. 20). The *VbIAciBau3967_0222* gene was the only one that responded to iron(III)-depleted conditions by showing a ~3-fold upregulation relative to control (no ferrichrome or iron(III) supplied into the growth media). For the other five response regulators no differences in the transcription levels in response to iron(III)-depleted conditions were detected.

Six response regulator genes, *VbIAciBau3967_1305, VbIAciBau3967_0222*, *VbIAciBau3967_1097*, *VbIAciBau3967_3515, VbIAciBau3967_3362,* and *bfmR* responded to iron(III)-abundant conditions. Two of these genes, *VbIAciBau3967_0222* and *VbIAciBau3967_1097*, were upregulated: ~2.5-fold and ~ 1.5-fold, respectively (Fig. 20).

The other four genes, *VbIAciBau3967_1305, VbIAciBau3967_3515, VbIAciBau3967_3362, bfmR* were downregulated in response to 100 μM Fe^{3+} (Fig. 20). Of the downregulated genes, *VbIAciBau3967_1305, VbIAciBau3967_3515* showed a ~ 2-fold downregulation, and the other two genes, *VbIAciBau3967_3362* and *bfmR*, showed a ~1.2-fold downregulation in response to iron(III)-rich conditions.

Remarkably, three of the six response regulator genes, *VbIAciBau3967_1305, VbIAciBau3967_3515* and *bfmR*, had their transcription levels elevated in response to iron(III)-depleted conditions relative to their transcription levels upon exposure to iron(III)-abundant

conditions (Fig. 20). The greatest difference, over 2-fold, between the transcription levels of the response regulator gene under iron(III)-rich and iron(III)-depleted conditions was detected for the *VbIAciBau3967_1305* gene.

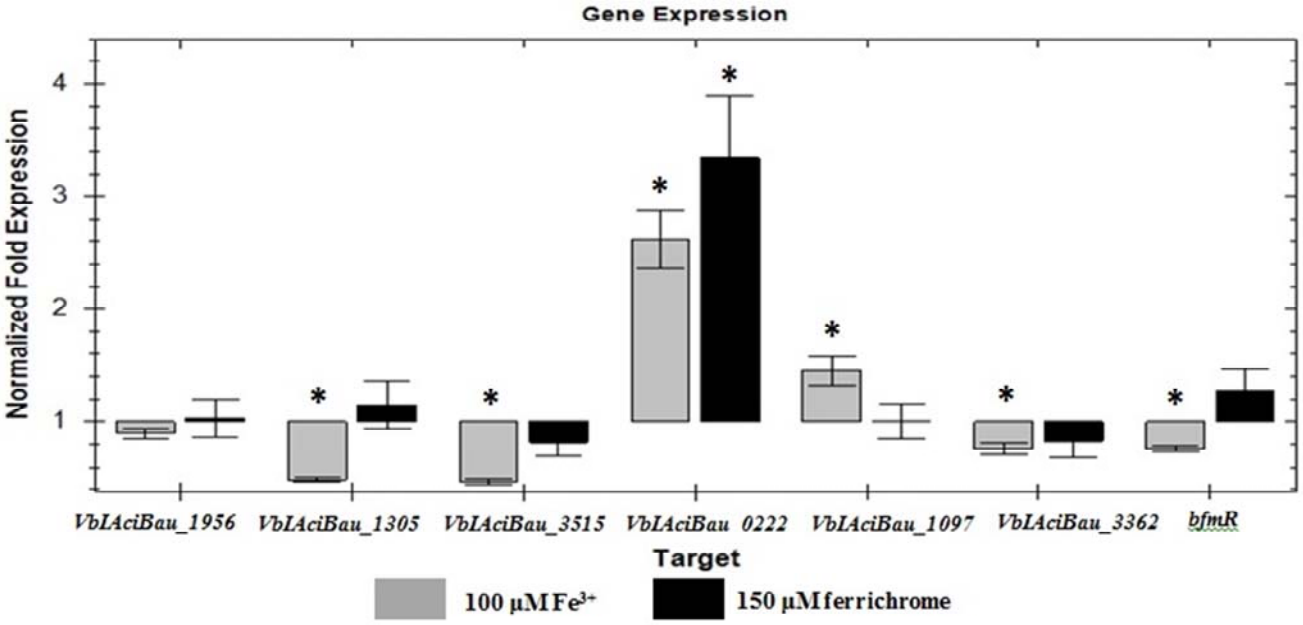

Figure 20. Comparative expression of the response regulators genes of the selected two-component systems in *A. baumannii* ATCC 19606 type strain upon exposure to iron(III)-abundant (100 µM Fe^{3+}) and iron(III)-depleted (150 µM ferrichrome) conditions (relative to control with no ferrichrome or iron(III) added to the media). The gene expression data is representative of two biological replicates. An asterisk indicates a statistically significant difference in the transcription levels of the target genes relative to control as determined by an unpaired Student's *t* test (with the 95% confidence interval). The error bars represent the standard deviation of the normalized fold expression values.

4. DISCUSSION

1. Effect of incubation temperature on transcription levels of RND efflux pumps and outer membrane porins

Previous research in *E. coli* showed that elevation of temperature above the standard incubation temperature (37°C) lead to an upregulation of the expression of RND efflux transporters, which could be contributing to development of the MDR bacterial strains (Alekshun and Levy, 1997). Heat shock was previously shown to promote antibiotic resistance to aminoglycosides in *A. baumannii* (Cardoso *et al*., 2010) as well as to adjust transcription of several antibiotic resistance determinants in *S. enterica* (Hartog *et al*., 2008). The findings lead to an assumption that exposure to higher than the optimal incubation temperatures may have an impact on transcription of the RND efflux systems and, possibly, some of the outer membrane proteins in *A. baumannii*.

This study revealed that the transcription levels of the *adeB* and *adeJ* genes were reduced in response to 30°C and 42°C incubation temperatures (except for *adeB* at 42°C, where the change in transcription was not shown to be statistically significant) in comparison to 37°C (Fig. 1). Interestingly, the transcription levels of the *adeB* and the *adeG* genes were found to be ~5-fold and ~2.8-fold, respectively, higher at 42°C in comparison to a suboptimal temperature of 30°C. The increased transcription of the *adeR* response regulator of the *adeABC* operon (~3-fold and ~2-fold at 30°C and 42°C, respectively) is not consistent with the observed downregulation of the *adeB* gene at 30°C (Fig. 1) and the unchanged transcription levels of the *adeB* gene at 42°C (Fig. 1). This discrepancy in transcription trends of the *adeR* response regulator and the gene that is a part of its regulon, the *adeB* gene, suggests that the additional, yet unknown, ways of the transcriptional control of the AdeABC efflux pump exist.

The elevated levels of transcription of the RND efflux genes at 42°C are consistent with the numerous reports in the literature. Adebusuyi and Foght (2011) reported a ~7-8-fold upregulation of the transcription levels of the *emhB* gene (part of the *emhABC* RND efflux operon in *P. aeruginosa*) in response to 35°C relative to 28°C. The same group also reported a 20-fold upregulation of transcription from the *emhABC* operon in response to 35°C when compared to its levels of transcription at 10°C.

Elsewhere, Hartog *et al*. (2008) reported elevated levels of transcription from the *acrAB* operon (encoding an inner membrane transporter and a membrane fusion component of the AcrAB-TolC RND efflux pump in *E. coli* and *Salmonella enterica*) at 37°C in comparison to 30°C in *S. enterica*. Additionally, this group has also reported upregulation of *micF* transcription, an antisense RNA that is known to be responsible for the downregulation of expression of the OmpF outer membrane protein (Delihas and Forst, 2001). Even though the downregulation of transcription of *ompF* was not shown, Hartog *et al*. have hypothesized that the observed upregulation of *acrAB*, together with the *micF* and *mar* operons may lead to development of a multidrug resistant phenotype.

The combination of the upregulation of transcription of RND efflux pumps (as one of the major mechanisms of drug extrusion from the bacterial cell) and a concomitant downregulation of transcription of outer membrane porins (as the major route of entry of the antimicrobials drugs inside the bacterial cell) is often suggested to have the potential for decreasing the bacterial susceptibility to antimicrobials. This very transcription pattern was observed in this study as the transcription levels of two RND efflux pump-encoding genes, *adeB* and *adeG*, were found to be elevated at 42°C when compared to their transcription levels at 30°C (Fig. 1). The elevated transcription levels of these RND efflux pumps were accompanied by a consistent

downregulation of the transcription levels of all three outer membrane porins: ~1.5 - 1.7-fold for the CarO and OprD-encoding genes at both 30°C and 42°C. The downregulation of the 33kDa porin-encoding gene at 42°C was found to be greater than that at 30°C: ~4-fold and ~1.3-fold, respectively (Fig. 1).

The results of the susceptibility assay of *A. baumannii* ATCC 19606 showed the decreased susceptibility of the organism at 42°C in comparison to 30°C only to one drug, ciprofloxacin: the diameter of the clearing zone decreased from 24 mm at 30°C to 22 mm at 42°C (Table 2). A slight decrease in the clearing zone, from 34.5 mm at 30°C to 34 mm at 42°C, was also observed for imipenem. For both gentamicin and ceftriaxone, the clearing zones at 30°C and 42°C increased from 11.5 mm to 12 mm and 12.5 mm to 15 mm, respectively, suggesting the increase in susceptibility of *A. baumannii* ATCC 19606 at higher temperatures. This finding is particularly interesting as ceftriaxone is a known substrate of the AdeABC efflux pump (Marchand *et al*., 2004). However, the increase of the transcription levels of the *adeB* gene at 42°C (relative to 30°C) was not reflected in the susceptibility of *A. baumannii* to this antimicrobial at 42°C (Table 2).

The discrepancy between the trends in the transcription levels of the major antibiotic resistance determinants and the antibiotic susceptibility of the host, *A. baumannii* ATCC 19606, could be due to the additional control over the protein levels of RND efflux pumps and outer membrane porins at the translational level. This may result in the effects of the observed transcriptional down/upregulation of the genes being negated. Kaatz and Seo (2004) reported over 4-fold downregulation of *norA* expression at 42°C relative to 30°C in *Staphylococcus aureus*. However, the indirect data obtained by determination of MICs to norfloxacin, a known NorA substrate, suggested that no significant change in the total levels of NorA protein in the

cell membrane occurred (Kaatz and Seo, 2004). Even though *norA* encodes an efflux pump that does not belong to the family of RND efflux transporters, this example clearly illustrates that the changes in levels of transcription of the gene will not necessarily be reflected in the levels of the protein (hence, phenotype) encoded by that gene.

Yet another factor possibly contributing to the discrepancies between the transcription levels of RND efflux pump- and outer membrane porin-encoding genes and the drug susceptibility of *A. baumannii*, could be a decrease in stability of mRNA at higher temperatures. Afonyushkin *et al.* (2003) reported no difference in the levels of OmpA transcript in *E. coli* in response to an increase in temperature from 28°C to 37°C. This group showed that the stability of mRNA was significantly decreased upon exposure to a higher temperature: from 13 minutes at 28°C down to 9 minutes at 37°C. Remarkably, it was proposed that the control at the translational level was likely to be responsible for compensation for the decreased mRNA stability at higher temperatures leading to protein levels remain unchanged.

It is possible that the decrease in stability of mRNA at higher temperatures together with the translational control were contributing to the discrepancies between the transcription patterns of these antibiotic resistance determinants and the susceptibility of the host, *A. baumannii* ATCC 19606, to the antibiotics tested.

2. Effect of iron(III) - depleted/repleted conditions on expression of RND efflux pumps and outer membrane porins

Iron(III) is an exceptionally important nutrient for bacteria due to its critical role in the energy balance of the cell, oxygen transport, oxidative stress response, DNA replication and regulation of gene expression (Scaar, 2010; Poole, 2012). Therefore, the process of iron(III)

sequestration plays an important part of human's immune response and is achieved via serum glycoprotein transferrin (association constant for iron(III) 10^{36} (Scaar, 2010) and lactoferrin, found in various secretions, such as saliva, tears, milk and nasal secretions.

In this study, the effect of iron(III) availability on expression of RND efflux systems in *A. baumannii* was shown for the first time. All three RND efflux genes were found to be downregulated upon exposure to iron(III)-rich medium in comparison to control. The *adeB* gene was found to be slightly upregulated (~1.4-fold), however, in response to iron(III)-depleted conditions (Fig. 4). Significant upregulation of the *adeR* response regulator, ~2.3-fold, was reported in response to iron(III)-limited condition (Fig. 5). This finding is consistent with the role of the AdeRS two-component system as a positive regulator of *adeABC* transcription.

Iron(III) regulation of transcription of the RND efflux pump-encoding operons is well-described in the literature. Poole *et al*. (1993) have reported the overproduction of the OprM protein, an outer membrane protein within the MexAB-OprM RND efflux pump, under iron (III)-limiting conditions that were achieved, similarly to this study, by supplying an iron(III) chelator, 2,2'-dipyridyl (instead of ferrichrome used in this study), into the growth medium.

Outer membrane porins are not believed to play any significant role in the direct iron(III) uptake from the environment into the bacterial cell as this role is generally attributed to small molecules known as siderophores (for example, acinetobactin in *A. baumannii*, Yamamoto *et al*., 1994; enterobactin in *E. coli*, Bleuel *et al*., 2005). However, several reports have shown the upregulation of the OprQ outer membrane porin in response to iron(III)–limited conditions in *P. aeruginosa* (Catel-Ferreira *et al*., 2012; Arhin and Boucher, 2010).

Therefore, due to the scarcity of information on regulation of the outer membrane porin's transcription in *A. baumannii*, the response of the *oprD* gene to iron(III) reported here poses a significant interest. A ~2-fold increase in the transcription levels of the *oprD* gene under iron(III)-depleted conditions (relative to iron(III)-rich conditions) shown in this study suggests that iron(III) plays a role in the *oprD* transcriptional regulation. Elsewhere, Arhin and Boucher (2010) have previously reported the upregulation of the OprQ outer membrane porin in response to iron(III)-limited conditions and suggested that this porin may be involved in enhancing *P. aeruginosa* ability to adhere to the extracellular matrix of its host, making OprQ an important virulence factor.

Remarkably, Catel-Ferreira *et al*. (2012) have recently shown that the OprD homologue in *A. baumannii*, despite belonging to the OprD subgroup of outer membrane proteins, is in fact more closely related to the OprQ protein of *P. aeruginosa.* This group of researchers showed the specificity of the OprD porin to extracellular iron(III) and suggested that this porin may play a role in adaption of the pathogen to iron(III) - and magnesium– depleted environment. It remains to be seen, however, whether the OprD porin plays the same role in *A. baumannii* virulence as does the OprQ porin in *P. aeruginosa*.

Elsewhere, Wei *et al*. (2006) have revealed a link between iron(III)-limitation and upregulation of the outer membrane protein OmpC in *Nitrosomonas europaea.* They have also suggested that OmpC may play a role in the uptake of iron(III) when this nutrient becomes scarce in the environment.

Remarkably, the *carO* gene was found to be consistently upregulated in response to both iron(III)-rich and iron(III)-depleted conditions (~1.8-fold and ~1.6-fold, respectively, Fig. 4).

The iron(III) control over transcription in Gram-negative bacteria is widely regarded to be mediated by the Fur protein, a transcriptional repressor that exerts its effect upon binding of the iron(III) ion followed by repression of transcription of the genes from the Fur regulon (Scaar, 2010; Poole, 2012). A drop of iron(III) levels leads to a Fe^{3+} dissociation from the Fur protein, subsequent dissociation of the Fur protein from the target gene's promoter and, hence, lifted repression of the target genes.

However, the upregulation of *carO* transcription under iron(III) – abundant conditions cannot be explained via the Fur-related pathway, where Fur acts as a repressor. Remarkably, Craig *et al*. (2011) have previously shown the upregulation of transcription of the *ompT* gene in *Vibrio cholerae* in response to Fe^{3+} in the growth medium. The transcriptional control of the *ompT* gene was shown to be mediated by the Fur protein serving in an unusual role of the positive regulator (Craig *et al*., 2011). Therefore, it is possible that that the Fur protein also serves as a positive regulator of transcription of the *carO* gene in *A. baumannii* ATCC 19606. If that is the case, then the drop in the transcription levels of the *carO* gene under iron(III)-depleted conditions (relative to iron(III)-abundant conditions) reported in this study (Fig. 4) may be explained by partial dissociation of the Fur protein from the *carO* promoter (in response to decreased Fe^{3+} levels in the media upon addition of the siderophore) and subsequent drop in the levels of transcription of the *carO* gene.

The results of the susceptibility assay of *A. baumannii* ATCC 19606 revealed decreased susceptibility of the organism to both imipenem and ciprofloxacin in response to iron(III)-rich conditions. The decrease of *A. baumannii* susceptibility to these drugs may be explained by the downregulation of expression of the 33kDa and OprD porin-encoding genes. Both of these outer membrane proteins are known to be involved in the uptake of carbapenems (imipenem). No

reports linking any of the three porins investigated in this study to the uptake of ciprofloxacin have been published to date.

3. Effect of ethanol shock on transcription of RND efflux pumps and porins

Numerous reports have suggested a link between ethanol exposure and *A. baumannii* pathogenicity towards *Dictyostelium discoideum* (Camarena *et al*., 2010), *Caenorhabditis elegans* (Smith *et al*., 2004) and *Saccharomyces cerevisiae* (Lamarche *et al*., 2008). It was of interest to see if exposure to ethanol affects the expression of the major antibiotic resistance determinants in the cell wall of *A. baumannii* such as RND efflux pumps and outer membrane porins.

The downregulation of transcription of both *adeJ* and *adeG* reported in this study (approximately 2-fold each in response to both 0.5% v/v and 1.1% v/v ethanol) is contradictory to the findings of Camarena *et al*. (2010) who did not report any changes in the transcription levels of these genes. In addition, Camarena *et al*. (2010) have reported a ~3-fold upregulation of expression of the *adeB* gene in response to 1.1% v/v ethanol - an effect we did not observe as the upregulation of *adeB* was not shown to be statistically significant (Fig. 6).

The discrepancies between these findings could be attributed to several factors. Primarily, it could be due to intrinsic differences between *A. baumannii* strains used: ATCC 19606 used in this study and ATCC 17978 used in the study by Camarena *et al*. (2010). In addition, this study had as its goal to look at the effect of short-timed ethanol shock on *A. baumannii*, as opposed to Camarena's study, where *A. baumannii* cells were grown in the presence of ethanol from the moment of inoculation of the growth media until the bacterial cells were harvested for mRNA

extraction. Lastly, different growth media, LB Lennox in our study and YPDA in the case of Camarena *et al*. (2010), were used.

Over 2.5-fold upregulation of *adeR* in response to both 0.5% v/v and 1.1% v/v ethanol was not consistent with the ~1.5-fold downregulation of *adeB* upon exposure of *A. baumannii* ATCC 19606 to 0.5% v/v ethanol (Fig. 6, 7). No statistically significant difference in the transcription levels of *adeB* in response to 1.1% [vol/vol] ethanol was observed in this study. These findings may be indicative of an additional mechanism of transcriptional regulation of the *adeABC* operon in addition to the AdeRS two-component system in *A. baumannii* ATCC 19606.

Transcriptional upregulation of RND efflux systems in response to ethanol exposure seems to be a common response in other Gram-negative organisms. For example, Ma *et al*. (1996) have reported the upregulation of transcription of the *acrAB* operon, encoding the AcrAB component of the AcrAB-TolC RND efflux system in *E. coli*, in response to general stress elicited by 4% ethanol.

Elsewhere, Fraud *et al*. (2008) have reported a ~1.5-fold upregulation of the MexCD-OprJ RND efflux pump in response to 1/8 MIC of ethanol. The same group has pointed at the link between the increased transcription from the *mexCD-oprJ* operon in *P. aeruginosa* and a decrease in the susceptibility to norfloxacin and erythromycin, two antibiotics that are known substrates of this pump. In contrast to our experiment, Fraud *et al*. had the bacterial cells exposed to ethanol for 2.5 hours (as opposed to a ~20 minutes exposure in our study that had its aim to look at the immediate impacts of ethanol on transcriptional regulation of the target genes) prior to the cells being harvested and subsequently prepared for qRT-PCR. This difference in ethanol exposure may account for the discrepancies between the results.

The differences in transcriptional responses of the MexCD-OprJ operon in *P. aeruginosa* and the AdeABC, AdeFGH and AdeIJK RND efflux systems in *A. baumannii* ATCC 19606 revealed in this study, are suggestive of the different roles that these efflux systems play in their respective hosts in response to membrane stress caused by ethanol. Remarkably, in addition to the ethanol impact on transcription of the MexCD-OprJ pump, Fraud *et al.* (2008) have also shown that this RND efflux system is inducible by some other membrane-damaging agents (MDA) such as detergents, biocides, some organic solvents and cationic antimicrobial peptides. Subsequently, Stickland *et al*. (2010) have shown that MexCD-OprJ is involved in the efflux of long-chain fatty acids further suggesting that this pump plays a role in restructuring of the damaged bacterial cell membrane of *P. aeruginosa* in response to MDAs such as ethanol, for example.

On the other hand, no particular role of any of the three RND efflux pumps in *A. baumannii* ATCC 19606 characterized to date, AdeABC, AdeIJK, AdeFGH, in the efflux of the cell membrane components has been reported to date. This difference in function may explain the discrepancy between the expression trends for the RND efflux genes reported in this study (all three were downregulated in response to ethanol shock, Fig. 6) and the MexCD-OprJ efflux pump of *P. aeruginosa* (Fraud *et al.,* 2008).

Since CarO has been shown to be involved in the transport of carbapenem antibiotics across the cell membrane (Catel-Ferreira *et al.,* 2011), we tested the susceptibility of *A. baumannii* ATCC 19606 to imipenem in the presence of 1.1% v/v ethanol. Consistent with the upregulation of *carO* upon exposure to 1.1% v/v ethanol, an increased susceptibility of *A. baumannii* ATCC 19606 to imipenem was observed: the diameter of the clearing zone increased

from 32 mm for control to 33.5 mm upon exposure of *A. baumannii* ATCC 19606 to 1.1% v/v ethanol (Table 2).

The increased susceptibility of *A. baumannii* ATCC 19606 to ceftriaxone in response to 1.1% v/v ethanol shock may also suggest that CarO is involved in the uptake of this third-generation cephalosporin antibiotic. The role of CarO in the uptake of β-lactam drugs in *A. baumannii* has never been reported to date. However, porins are known to mediate the transport of β-lactams in other Gram-negative organisms. For example, reduced expression of *ompF* was shown to lead to increased levels of the β-lactam resistance in *E. coli* (Jaffe *et al*., 1983). This claim needs further investigation (e.g., gene-knockout studies), however, to definitively prove the direct involvement of the CarO outer membrane protein in the uptake of β-lactams (cephalosporins and ceftriaxone, specifically) in *A. baumannii*. It is also worth mentioning that the downregulation of the *adeIJK* and *adeFGH* operons in response to 1.1% v/v ethanol is unlikely to be the reason behind the increased susceptibility of *A. baumannii* to ceftriaxone as this antibiotic is not known to be a substrate of these RND efflux systems in *A. baumannii*.

4. Effect of oxidative stress on transcription of RND efflux pumps and porins

Oxidative stress is one of the most important environmental stresses a bacterial pathogen can be exposed to. A wide diversity of reactive oxygen species is being generated both outside (e.g., ROS produced by host immune system, phagocytes) and inside of the bacterial pathogen (e.g., ROS generated due to respiratory reactions of any aerobic organism; ROS generated upon exposure of the pathogen to certain representatives of bactericidal antibiotics). Similarly, several reports have exposed the link between the recruitment of the antioxidant mechanisms in response to exposure of bacteria to certain antibiotics that are known to generate ROS, which, in turn, are

responsible for the bactericidal effects of these antibiotics (Dwyer *et al*., 2009). As a part of the adaptive responses to environmental stress, the regulation of RND efflux systems and outer membrane porins, as the major intrinsic determinants of antibiotic resistance, were investigated in this study.

The levels of transcription of all three RND efflux pumps characterized in *A. baumannii* to date were found to be increased in response to oxidative stress (Fig. 9). These findings are consistent with the previously reported sensitivity of the representatives of the RND family of transporters to oxidative stress in several Gram-negative organisms such as *E. coli* and *P. aeruginosa* (Chen *et al*., 2008; Fabrega *et al*., 2010; Miller and Sulavik, 1996; Fraud and Poole, 2011).

In *P. aeruginosa* Chen *et al*. (2008) have shown the transcriptional upregulation of the *mexAB-oprM* RND efflux operon (assessed, similarly to this study, by the transcription levels of the *mexB* gene, encoding an inner-membrane transporter) in response to oxidative stress caused by hydrogen peroxide. The observed effect on transcription of the *mexAB-oprM* RND efflux operon was attributed to oxidation of the MexR-repressor, a MarR (***m***ultiple ***a***ntibiotic ***r***esistance) family of regulatory proteins (Alekshun and Levy, 1997), and subsequent alleviation of transcriptional repression of the *mexAB-oprM* operon. In accordance to these results, Fabrega *et al.* (2011) have shown the AcrAB-TolC RND efflux system from *E. coli* to be under the transcriptional control of the SoxR repressor within the SoxRS two-component system, which, in its turn, is known to mediate the oxidative responses in *E. coli* (Miller and Sulavik, 1996). Exposure to oxidative stress causes oxidation of the SoxR repressor, its dissociation from DNA and subsequent transcriptional activation of the *acrAB-TolC* operon (Miller and Sulavik, 1996; Poole, 2012).

Elsewhere, Fraud and Poole, (2011) reported the induction of the *mexXY* genes of the MexXY-OprM RND efflux pump of *P. aeruginosa* in response to both oxidative and nitrosative stresses. Remarkably, the observed response was found to be mediated via induction of the *PA5471* gene, previously shown to play a role in antimicrobial induction of the MexXY components in *P. aeruginosa* (Morita *et al*., 2006). Interestingly, the *mexXY* genes were found to be also upregulated in response to antibiotics (aminoglycosides, specifically) that are known to target ribosomes and the regulation was shown to occur via the same *PA5471* gene. It was suggested that aberrant proteins produced by the bacterial cell upon disruption of ribosomes act as the triggers for upregulation of transcription of the *mexXY* operon and that the MexXY-OprM RND pump might be involved in the efflux of these damaged proteins outside of the cell (Fraud and Poole, 2011; Poole, 2012).

Involvement of the RND efflux pumps characterized in *A. baumannii* to date in the efflux of aberrant proteins has been never reported before. However, based on the results of this study together with the previous reports in other Gram-negative organisms, it would be of interest to investigate a possible involvement of the AdeABC, AdeIJK and AdeFGH pumps in the efflux of aberrant proteins in response to oxidative stress in *A. baumannii*.

A ~2.5-fold and a ~2.9-fold upregulation of transcription of the *adeR* gene in response to 1 mM and 2 mM H_2O_2, respectively, was revealed (Fig. 10). This result is consistent with the increase of the *adeB* transcription levels reported above.

No reports to date have looked into the details of transcriptional regulation of any of the three porins studied here in response to oxidative stress in *A. baumannii* or any other Gram-negative organisms. However, the downregulation of the *carO* and *33kDa* genes (in response to

1 mM and 2 mM H_2O_2, respectively) reported in this study is consistent with the findings reported by Calderon *et al*. (2011) who showed the downregulation of another porin-encoding gene, *ompD*, in response to H_2O_2 in *Salmonella enterica.* This finding may suggest that the CarO and 33kDa outer membrane porins, similarly to OmpD of *S. enterica* (Calderon *et al*., 2011), may play some role in facilitating the uptake of hydrogen peroxide in *A. baumannii* ATCC 19606.

Simultaneous upregulation of *adeB* and downregulation of *carO* in response to oxidative stress may potentially lead to a decrease in the susceptibility of the pathogen to carbapenems. That is because these outer membrane proteins have been explicitly shown to play a role in the efflux and influx, respectively, of the carbapenem class of antibiotics in *A. baumannii* (Mar Tomas *et al*., 2005; Catel-Ferreira *et al*., 2011; Mussi *et al*., 2007; Siroy *et al*., 2005).

5. Effect of salicylate exposure on expression of RND efflux pumps and outer membrane porins

The impact of salicylate, a common component of antipyretic drugs, on expression levels of the major intrinsic antibiotic resistance determinants has never been investigated in *A. baumannii*. In general, however, the impact of salicylate on antibiotic resistance in bacteria has been associated mostly with altered expression of efflux systems and outer membrane porins ultimately affecting the drug accumulation inside the bacterial cell (Price *et al*., 2000).

The downregulation of the transcription levels of the RND efflux pumps (~1.5-fold and ~2.5-fold in response to 2.5 mM and 4 mM of salicylate, respectively, in the case of the *adeB* gene) is contradictory to the data published in the literature. Exposure of various Gram-negative pathogens to salicylate has been widely shown to upregulate transcription of RND efflux pumps

(Ma *et al*., 1995; Cohen *et al*., 1989; Hartog *et al*., 2010). Along with the downregulation of *adeB*, both in response to 2.5 mM and 4 mM sodium salicylate (Fig. 11), however, it was also found that *adeR* was ~2.5-fold upregulated in response to 2.5 mM salicylate but ~2-fold downregulated in response to 4 mM salicylate (Fig. 12). The discrepancies between the transcription trends of *adeB* and *adeR* suggest that an additional mechanism of regulation of the *adeABC* operon may exist in *A. baumannii* ATCC 19606.

The upregulation of the AcrAB-TolC RND efflux pump in response to salicylate was previously reported in *E. coli* (Ma *et al*., 1995) and in *Salmonella enterica* (~3.4-fold) in response to 5 mM salicylate (Hartog *et al*., 2010). Hannula and Hanninen (2008) and Shen *et al*. (2011) also showed a ~2-fold and a ~3-fold increase in expression of the *cmeABC* operon in *Campylobacter jejuni* (similar to this study, assessed by quantifying the transcription levels of the inner-membrane transporter-encoding gene, *cmeB*) in response to 100 μg/mL (0.625 mM) and 200 μg/mL (1.25 mM) sodium salicylate, respectively.

In general, the mechanism of regulation of RND efflux pumps by salicylate has been shown to occur in *E. coli* via a *marRAB* operon, where *marR* encodes a transcriptional repressor of the *marRAB* operon and MarA serves as a transcriptional activator of the *marRAB* regulon (Price *et al*., 2000). Salicylate is known to bind to the MarR regulator, hence, preventing binding of the repressor to a *marO* operator within the *marRAB* operon. This event leads to overexpression of the MarA activator and subsequent transcriptional upregulation of the genes within the *marRAB* regulon, amongst which is the AcrAB-TolC RND efflux pump (Price *et al*., 2000; Cohen *et al*., 1993).

Similarly to a *mar*-dependent overexpression of RND efflux genes in *E. coli*, salicylate was also shown to upregulate the *emrAB* operon which is under the control of the EmrR repressor. In addition, the EmrR repressor has been shown to share sequence similarity with the MarR protein discussed above (Lomovskaya *et al*., 1995). Similar mechanism of action of salicylate was also reported in *Campylobacter jejuni,* where salicylate was shown to bind and inactivate CmeR, a transcriptional repressor of the TetR family that controls expression of the CmeABC RND efflux pump. Binding of salicylate to CmeR precludes binding of the repressor to the *cmeABC* promoter (Shen *et al*., 2011; Lei *et al*., 2011).

Elsewhere, Nair *et al*. (2004) reported a ~2-fold and a ~4-fold upregulation of the *ceoAB-opcM* RND efflux operon in *Burkholderia cepacia* in response to 2 mM and 5 mM salicylate, respectively. Interestingly, upon exposure of *A. baumannii* ATCC 19606 to the concentrations of salicylate above 4 mM (5 mM and 10 mM, specifically) in this study, the transcription levels of the housekeeping genes tested (16S rRNA-encoding gene and *fmcC*) were found to be significantly fluctuating relative to the baseline conditions (no exogenous salicylate added) making qRT-PCR analysis unreliable. The changes in the transcription levels of *adeG* and *adeJ* in response to salicylate were not found to be statistically significant.

The downregulation of the RND efflux pumps reported in this study (up to 2.5-fold for the *adeB* gene, Fig. 11) in response to salicylate exposure may suggest a *mar*-independent mode of regulation of the RND efflux pumps by salicylate. Even though downregulation of RND efflux pumps in Gram-negative organisms in response to salicylate has never been reported before, Cohen *et al*. (1993) have previously suggested the existence of a mode of regulation of RND efflux pumps that is alternative to a *mar*-dependent mechanism described above.

The mechanism of regulation of expression of outer membrane porins in Gram-negative bacteria by salicylate is very poorly investigated. The only known mode of regulation was reported to occur in *E.coli* via the anti-sense *micF* RNA transcripts (Rosner *et al*., 1991). No reports discussing the regulation of porins in *A. baumannii* has been published to date.

The downregulation of the OprD-encoding gene, to a maximum of 2-fold in response to 2.5 mM salicylate (Fig. 11), is consistent with the previously published findings that exposed the link between salicylate exposure and outer membrane porin downregulation. Ochs *et al*. (1999) have reported reduced expression of the OprD-encoding gene of *P. aeruginosa* in response to salicylate. The downregulation of the OmpF porin of *E. coli* in response to a range of salicylate concentrations in the growth media (0.5-10 mM) was shown by Rosner *et al*. (1991), while Sawai *et al*. (1987) have also reported a slight reduction of the OmpC levels upon exposure to salicylate. The elucidated mechanism of downregulation of the OmpF transcript levels was shown to occur via upregulation of the *micF* transcripts, the anti-sense RNA molecules to *ompF* transcript (Rosner *et al*., 1991). In accordance with *E. coli* data, Puig *et al*. (1995) have also found that exposure of *S. marcescens* to 3 mM salicylate resulted in a completely abolished or severely repressed synthesis of the Omp3 porin.

The repression of synthesis of certain outer membrane proteins in response to salicylate exposure has been also widely reported in *Pseudomonas* sp. (Burns and Clark, 1992; Sawai *et al*., 1987; Ochs *et al*., 1999) but the mechanism of action remains largely unclear to this date.

The results of the susceptibility assay of *A. baumannii* ATCC 19606 were quite contradictory to the gene expression data. The downregulation of the OprD-encoding gene did not translate into a decreased susceptibility of the organism to imipenem antibiotic that uses the

OprD outer membrane porin as its main route of entry into the bacterial cell. On the contrary, an increased susceptibility of *A. baumannii* ATCC 19606 to imipenem upon exposure to 4 mM sodium salicylate was observed as the diameter of the clearing zone increased from 32 mm to 34.5 mm (Table 2). Interestingly, transcriptional downregulation of the *adeB* gene did not translate into an increased susceptibility of the organism to ciprofloxacin antibiotic, a known substrate of the AdeABC efflux pump: the diameter of the clearing zone decreased from 21.5 mm to 19.5 mm upon exposure to 4 mM salicylate, suggesting a decrease in susceptibility. However, the increased levels of resistance of *A. baumannii* ATCC 19606 to ciprofloxacin may be explained by the emergence of resistant mutants. Fluoroquinolone antibiotics were widely reported to increase the mutation frequency in *S. aureus* leading to an elevated resistance to fluoroquinolones and fusidic acid (Gustafson *et al*., 1999; Price *et al*., 1999).

6. Effect of steady-state osmotic stress on expression of RND efflux pumps and outer membrane proteins

Steady-state osmotic stress is associated with bacterial responses that aim to contain the integrity of the cell membrane. No studies have looked into the transcriptional response of *A. baumannii* to changes in osmolarity of the growth media. According to our data, exposure of *A. baumannii* ATCC 19606 to steady-state osmotic stress results in the downregulation of expression of the *adeB* gene as well as all three outer membrane protein-encoding genes analyzed in this study (the 33-kDa outer membrane protein-encoding gene, *oprD* and to a smaller extent *carO*).

With respect to RND efflux pump regulation, the downregulation of the *adeB* gene in response to 0.3 M (Fig. 14) sucrose contradicts previously published studies in other Gram-

negatives. Giuliodori *et al.* (2007) have shown that high osmolarity of the medium was responsible for an increase in expression of the *rpoS* and *cpxR* transcription factor-encoding genes, which subsequently resulted in the upregulation of expression of the AcrAB-TolC RND efflux pump in *E. coli*. Remarkably, over 6-fold upregulation of the *adeR* regulator (Fig. 15) in response to 0.2 M sucrose did not result in the upregulation of the *adeB* gene as was anticipated due to the AdeR protein being a positive regulator of the *adeABC* operon. However, a well-pronounced decrease in the transcription levels of the *adeB* gene in response to 0.3 M sucrose relative to 0.2 M sucrose is consistent with a drastic drop (~2.5-fold) in the transcription levels of the *adeR* regulator in response to 0.3 M sucrose (Fig. 14, 15).

While the impact of osmolarity on expression of outer membrane proteins in various Gram-negative species is quite well documented, no studies that investigated the effect of steady-state osmotic stress on transcriptional regulation of the outer membrane proteins in *A. baumannii* have been published to date. Villarejo *et al*. (1983) found that the production of the OmpF outer membrane protein in *E. coli* grown in the high osmolarity media (20% sucrose used by Villarejo *et al.* corresponds to ~ 0.6 M sucrose) is greatly reduced when compared to the OmpF levels under low osmolarity conditions (nutrient broth with no sucrose added). However, under the same conditions of high osmolarity the levels of expression of another important outer membrane protein, OmpC, have been shown to increase. Interestingly, upon exposure of *A. baumannii* ATCC 19606 to the concentrations of sucrose above 0.3 M (0.4 M and 0.5 M, specifically) the transcription levels of the housekeeping genes tested (16S rRNA-encoding gene and *fmcC*) were found to be fluctuating relative to the baseline conditions (no exogenous sucrose supplied into the growth media) making qRT-PCR analysis unreliable.

This mode of regulation of porin production may explain the increased transcriptional downregulation of the 33-kDa porin upon exposure to 0.3 M sucrose relative to 0.2 M sucrose (Fig. 14). Besides, this may also explain the regulation of the *carO* gene observed here, which was found to be downregulated ~1.2-fold upon exposure to 0.3 M sucrose when compared relative to its transcription levels under 0.2 M sucrose (Fig. 14). To fully answer the question regarding the details of porin transcription regulation in *A. baumannii* in response to steady-state osmotic stress, the identification and characterization of two-component systems potentially involved in the steady-state osmotic stress response of *A. baumannii*, as well as the patterns and the levels of phosphorylation of their response regulators, are required. Overall, the downregulation of transcription of the 33-kDa outer membrane protein-encoding gene and *oprD*, may potentially have a significant effect on resistance of *A. baumannii* ATCC 19606 to carbapenems as both porins are known to be the main route of entry of this family of antibiotics into *A. baumannii* cells.

7. Effect of blue light on expression of RND efflux pumps and outer membrane porins

A few reports have previously shown that a response to blue light can be involved in regulation of a number of the bacterial functions: stress response, biofilm formation and virulence (Gaidenko *et al*., 2006; Tschowri *et al*., 2009). It was of interest to test if exposure to blue light had any effect on the expression levels of RND efflux pumps and outer membrane porins in *A. baumannii.*

No changes in the expression levels of two RND efflux pumps (encoded by the *adeB* and *adeG* genes) and all three outer membrane porins (encoded by the *33kDa, carO* and *oprD* genes) was detected in *A. baumannii* ATCC 19606 in response to blue-light exposure. The *adeG* gene

showed a ~1.3-fold downregulation in response to blue-light exposure relative to dark conditions. No reports linking transcription of RND efflux systems and/or outer membrane porins in Gram-negatives to exposure to blue-light has been published to date.

8. Comparative expression of two-component systems in *A. baumannii* ATCC 19606 type strain and *A. baumannii* clinical isolates, AB030 and AB031

The role of two-component systems (TCS) in bacterial antibiotic resistance, strain virulence and potential for biofilm formation has been known for a while and certain representatives of TCS have been well characterized in the literature. In *A. baumannii*, the PmrAB system has been shown to be involved in resistance to colistin (Adams *et al*., 2009). The exact mechanism and/or the downstream targets of the PmrA protein that are involved in the colistin resistance in *A. baumannii*, however, have not been elucidated to date. It is speculated that the downstream targets of the PmrAB TCS in *A. baumannii* may be involved in the modification of the lipid A components of the outer membrane lipopolysaccharide, a known target of colistin (Paulus and Gray, 1964; Vaara and Viljanen, 1985). Yet another TCS in *A. baumannii, bfmRS,* is known to be able to sense some of the key signals in the extracellular space (e.g., cell density, presence/absence of a variety of nutrients and cations) that trigger the pili assembly and the surface-adhesion protein production – the processes involved in biofilm formation (Tomaras *et al*., 2008).

This study, without going into the details of any of the selected two-component systems in *A. baumannii*, showed the difference in the transcription levels of the TCS response regulator genes between *A. baumannii* type strain, ATCC 19606, and two clinical isolates of *A. baumannii*, AB030 and AB031 (a MDR and a biofilm-forming strain, respectively). It was of interest to see

if the selected TCS were in fact differentially expressed between two clinical isolates showing distinct phenotypes and a type strain of *A. baumannii*, ATCC 19606. The TCS genes that are differentially expressed between clinical isolates and a type strain of *A. baumannii* can be selected as potential candidates for knockout studies to elucidate the role of such genes in the development of the MDR phenotype and/or biofilm formation by *A. baumannii*.

Two-component systems have been also linked to bacterial resistance to non-antibiotic compounds. The ArcAB TCS in *E. coli* was shown to play a key role in bacterial resistance to reactive oxygen species, possibly via the upregulation of the amino acid and protein synthesis and assimilation (Loui *et al*., 2009). Elsewhere, Stincone *et al*. (2011) have shown the importance of the OmpR-EnvZ two-component system regulator in the resistance of *E. coli* to acid stress.

Seven TCS response regulators of ten investigated, were found to be slightly downregulated in both clinical isolates of *A. baumannii* relative to the type strain, ATCC 19606, with just one regulator, *VbIAciBau3967_1097*, being ~4-fold upregulated in AB030. This finding makes *VbIAciBau3967_1097* a potential candidate for further studies to elucidate its role in the MDR phenotype of the AB030 clinical isolate. Surprisingly, our data on the *bfmRS* TCS failed to support previously published results linking the regulation of biofilm formation in *A. baumannii* to the *bfmRS* operon: transcription of the *bfmRS* operon was over 10-fold downregulated in AB031, a biofilm-forming clinical isolate (Fig. 19).

Overall, several selected TCS response regulators showed differential transcription between the type strain and the MDR and biofilm-forming clinical isolates of *A. baumannii.*

These findings hint at a possible role these TCS have to play in the development of the MDR and biofilm formation, making them good candidates for knockout studies.

9. Comparative expression of two-component systems under iron(III)-depleted and iron(III)-repleted conditions

With iron(III) playing a very important role as a co-factor for numerous bacterial enzymes and being a key player in cell respiration, levels of iron(III) in extracellular medium are vigorously monitored by the bacterial cell. Interestingly, a drop of iron(III) levels is believed to be used by a bacterial pathogens as a signal of entry into their hosts (Scaar, 2010). The stress response to iron(III) starvation involves the upregulation of the cell surface receptors and transport proteins that are directly involved in the free iron(III)- binding, as well as the upregulation of siderophores, small, high affinity iron(III)-chelating molecules that compete with the host iron(III)-sequestering proteins for this essential nutrient (Escolar *et al*., 1999; Daniel *et al*., 1999; Scaar, 2010; Miethke and Marahiel, 2007).

The response generated by the bacterial cell to enhance its chances of acquisition of required levels of iron(III) is the final step of a signaling cascade, activated upon a drop of iron(III) levels in the extracellular space. The Fur protein is a global repressor well-known to be involved in the transcriptional control of the genes involved in the siderophore synthesis, transport, secretion as well as the uptake of the siderophore-sequestered iron(III) ions in different Gram-negatives (Escolar *et al*., 1999; Daniel *et al*., 1999; Scaar, 2010; Miethke and Marahiel, 2007).

In addition to Fur, a global regulator, siderophore utilization is also known to be controlled by the "minor" regulators, such as two-component systems. The alternative sigma

factors and the AraC-type regulators are the other two groups of regulators shown to be involved in the iron(III) starvation response (Miethke and Marahiel, 2007). No such TCS have been identified in *A. baumannii* to date but have been found in other clinically relevant Gram-negative pathogens. These sensory systems may be under a direct downstream control of the Fur repressor or can be activated directly by induction by other players in the iron(III) starvation response. For instance, the PfeR-PfeS two-component system of *P. aeruginosa* has been shown to be involved in the induction of the biosynthesis of the ferric enterobactin receptor PfeA, hence, assisting the cell in the iron(III) uptake (Dean *et al*., 1996).

This study showed that the transcription levels of at least three response regulators, *VbIAciBau3967_1305, VbIAciBau3967_3515* and *bfmR*, were upregulated under iron(III)-depleted conditions (150 μM ferrichrome) when compared to their levels under iron (III) rich conditions (100 μM Fe^{3+}) (Fig. 20). However, when expression of these response regulators was compared to standard conditions of LB Lennox (without any additional Fe^{3+} and/or ferrichrome supplied into the growth media), the levels of transcription of all three response regulators discussed above failed to show any significant difference (Fig. 20). Therefore, these three response regulators are good candidates for further investigation with respect to their role in *A. baumannii* stress responses to iron(III) limitation. Interestingly, one of the response regulators of the seven studied, *VbIAciBau3967_0222*, was significantly upregulated under iron(III)-limited as well as iron(III)-rich conditions (Fig. 20). This is the first report investigating the role of two-component systems in *A. baumannii* response to iron(III) starvation.

5. FUTURE DIRECTIONS

A QUANTIFICATION OF PROTEIN LEVELS OF RND EFFLUX PUMPS AND OUTER MEMBRANE PORINS IN RESPONSE TO ENVIRONMENTAL STRESS CONDITIONS

While it was shown that transcription levels of the RND efflux pumps and outer membrane porins are subject to change in response to different environmental conditions, the next steps would be to test how the changes in transcription levels correlate with the protein levels of the AdeABC, AdeFGH and AdeIJK RND efflux pumps and the 33kDa, CarO and OprD outer membrane porins in *A. baumannii* ATCC 19606.

B PREPARING KNOCK-OUTS OF RESPONSE REGULATORS OF SELECTED TWO-COMPONENT SYSTEMS

It was shown that *A. baumannii* ATCC 19606 regulates the levels of the response regulators of selected two-component systems in response to iron(III) levels. In addition, it was found that transcription levels of certain response regulators differ between *A. baumannii* type strain, ATCC 19606, and two clinical isolates, AB030 and AB031. The future study should look into the development of the genetic tools needed for generation of the knock-outs of the most responsive TCS. This will allow to investigate the role of these TCS in clinically relevant phenotypes of *A. baumannii.*

C FURTHER INVESTIGATION INTO THE ROLE OF TCS IN THE ADAPTIVE RESPONSE TO ENVIRONMENTAL STRESS CONDITIONS

It poses a particular interest to look into the role that identified TCS play in response to different environmental stress conditions investigated in this study as well as to any other major environmental stresses. This approach can be combined with the knock-outs generated as was described in the previous subsection.

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APPENDIX A. Standardization of primer sets for qRT-PCR reactions

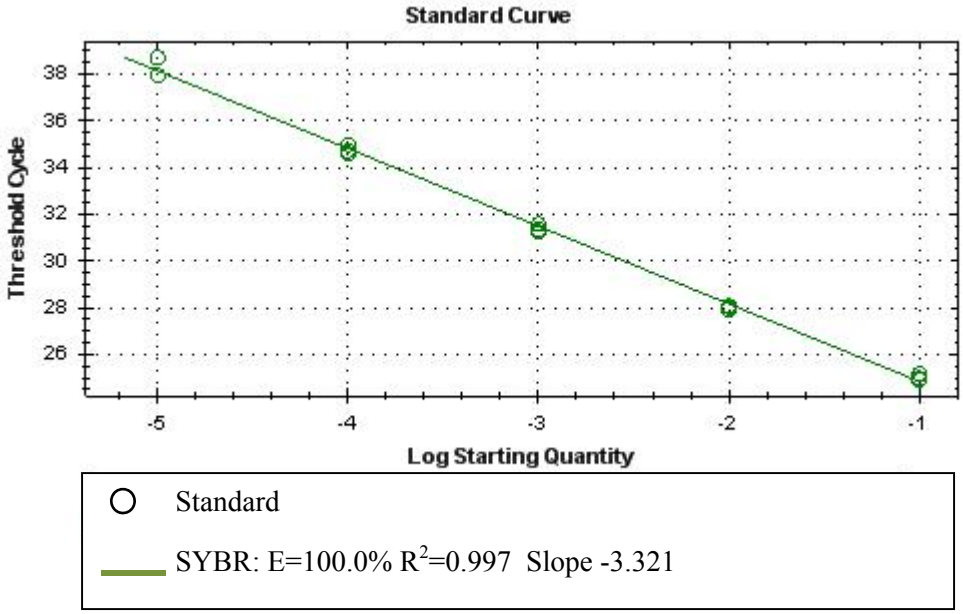

Figure 1. Standardization of primers for the *VbIAciBau3967_1956* gene.

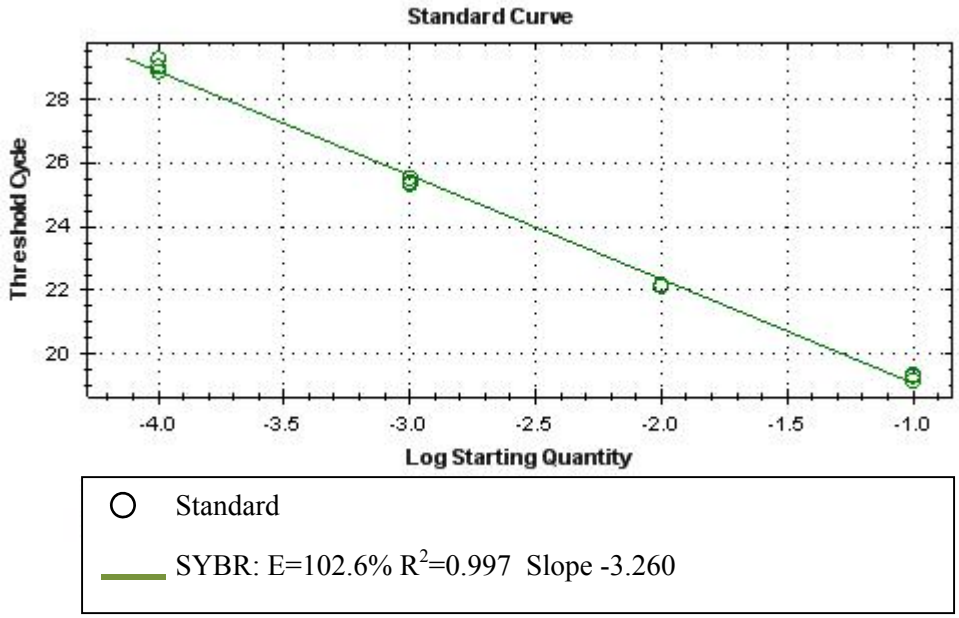

Figure 2. Standardization of primers for the *VbIAciBau3967_1305* gene.

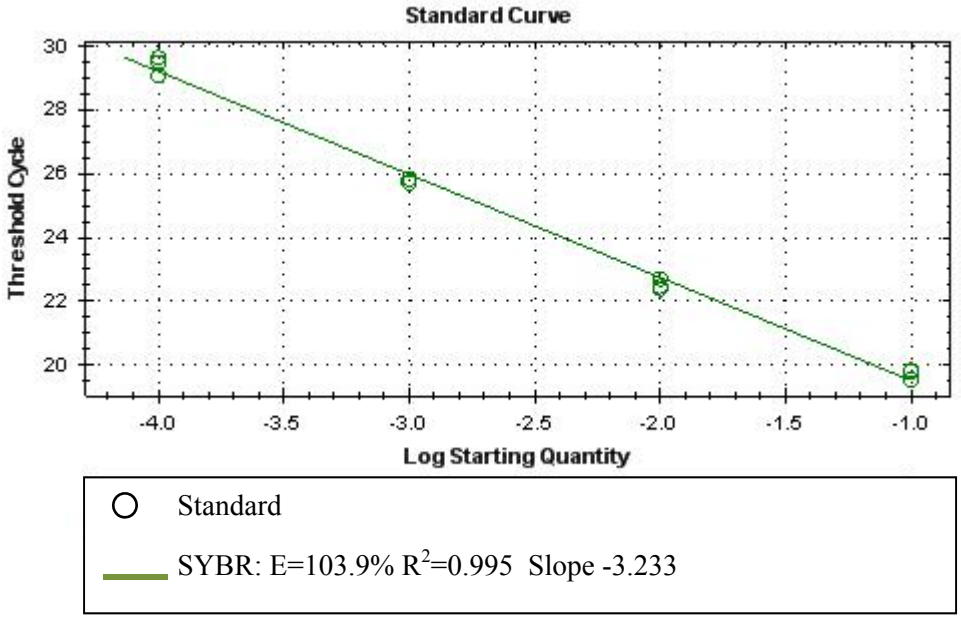

Figure 3. Standardization of primers for the *VbIAciBau3967_3515* gene.

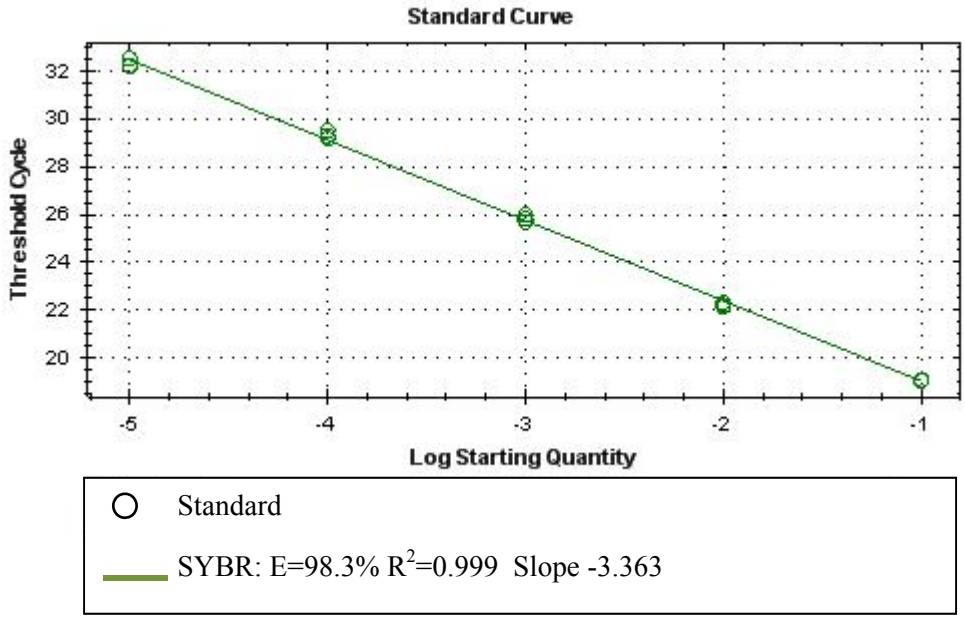

Figure 4. Standardization of primers for the *VbIAciBau3967_0658* gene.

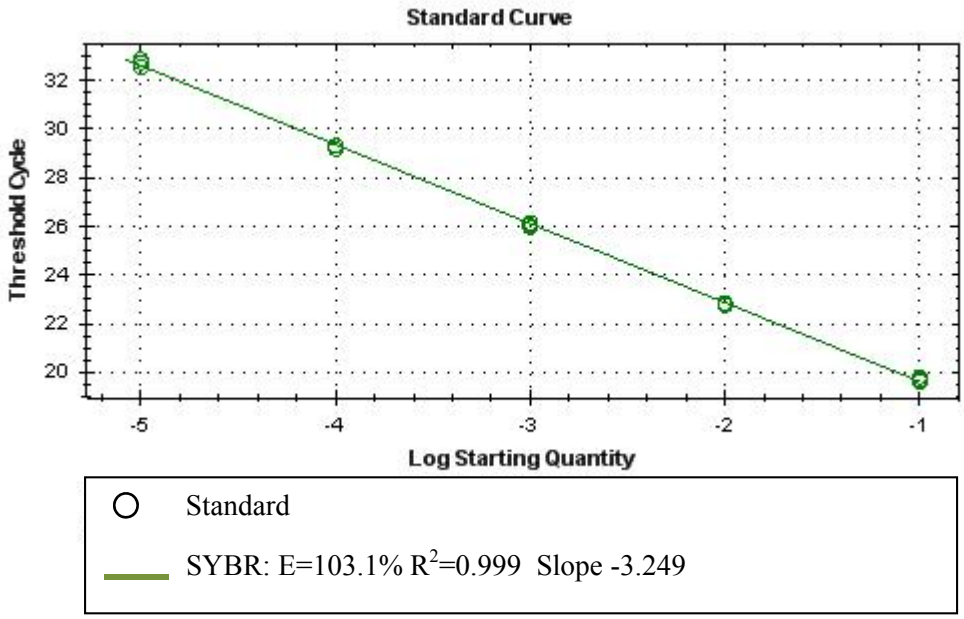

Figure 5. Standardization of primers for the *VbIAciBau3967_0222* gene.

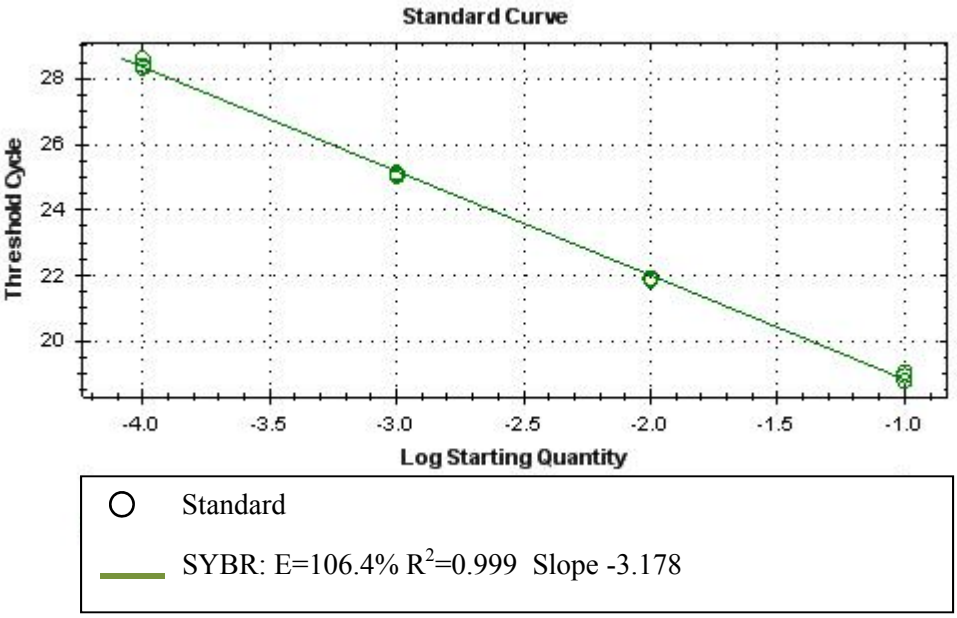

Figure 6. Standardization of primers for the *VbIAciBau3967_1128* gene.

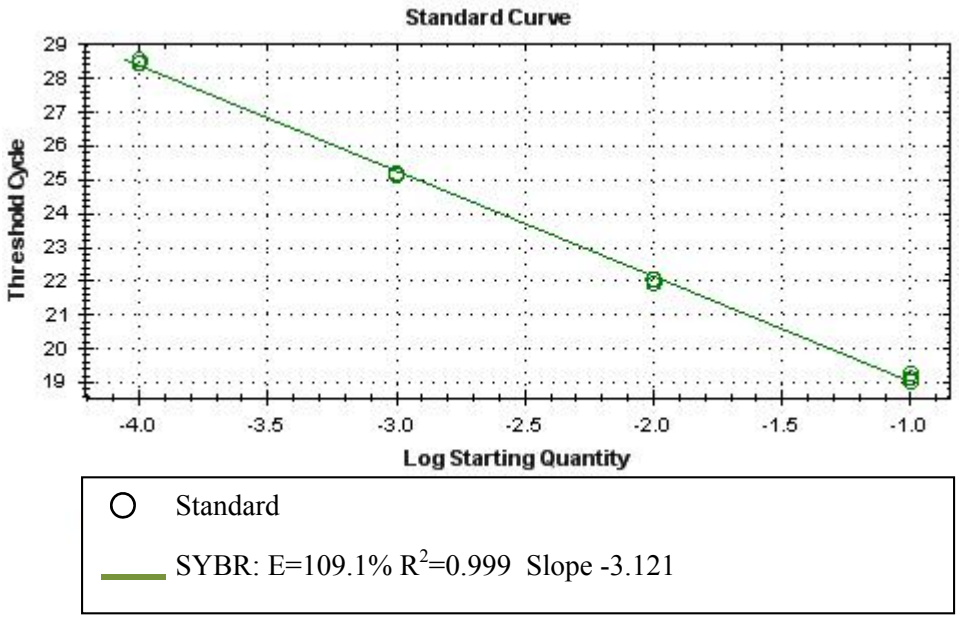

Figure 7. Standardization of primers for the *VbIAciBau3967_1097* gene.

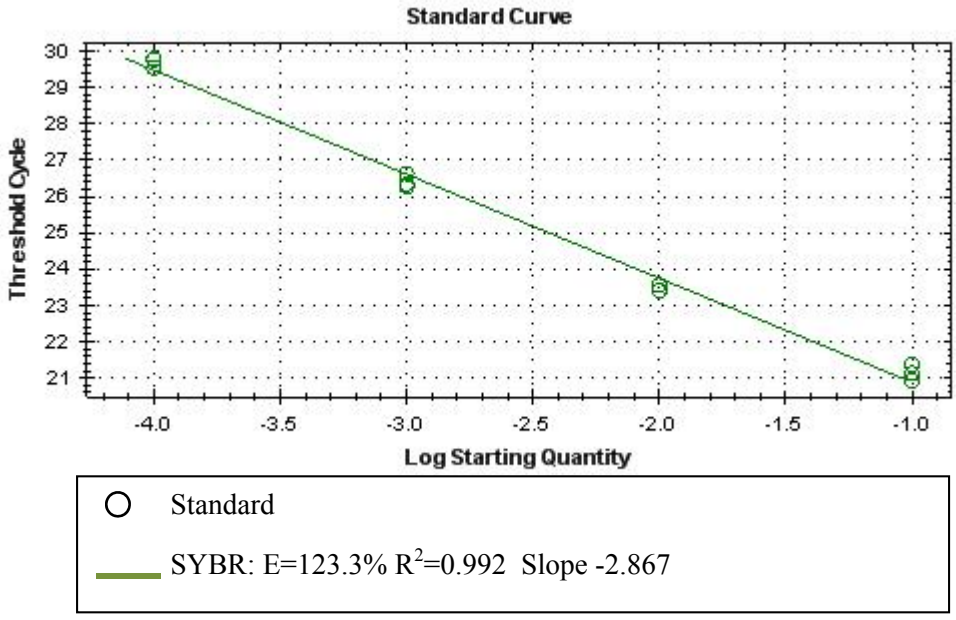

Figure 8. Standardization of primers for the *VbIAciBau3967_0991* gene.

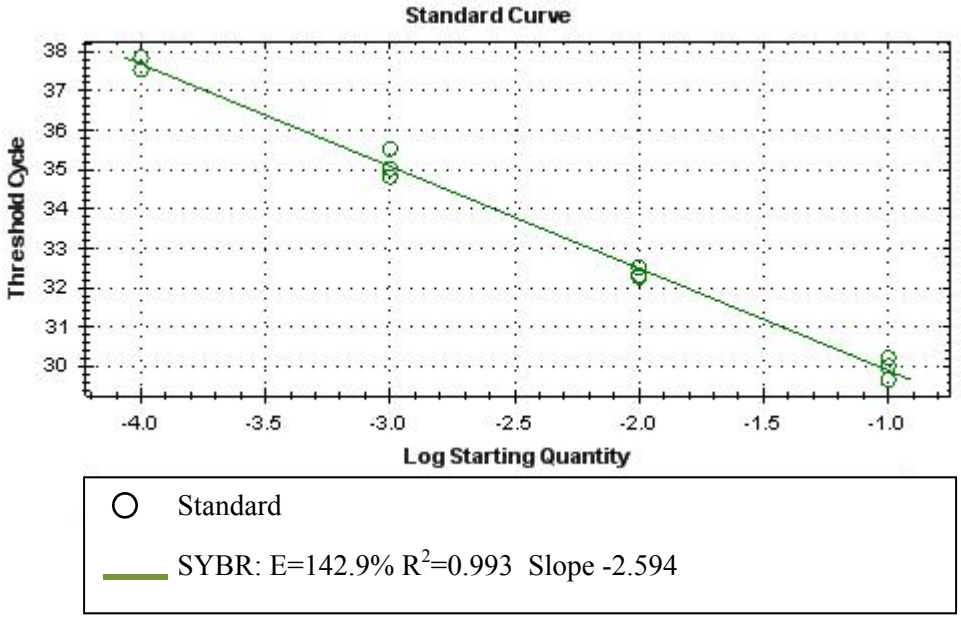

Figure 9. Standardization of primers for the *VbIAciBau3967_0480* gene.

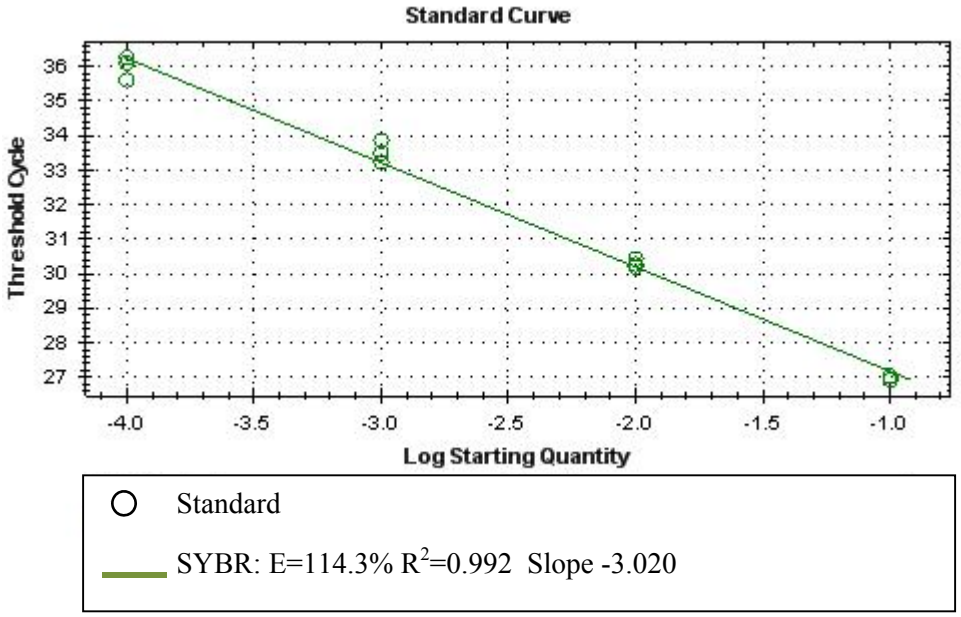

Figure 10. Standardization of primers for the *VbIAciBau3967_2809* gene.

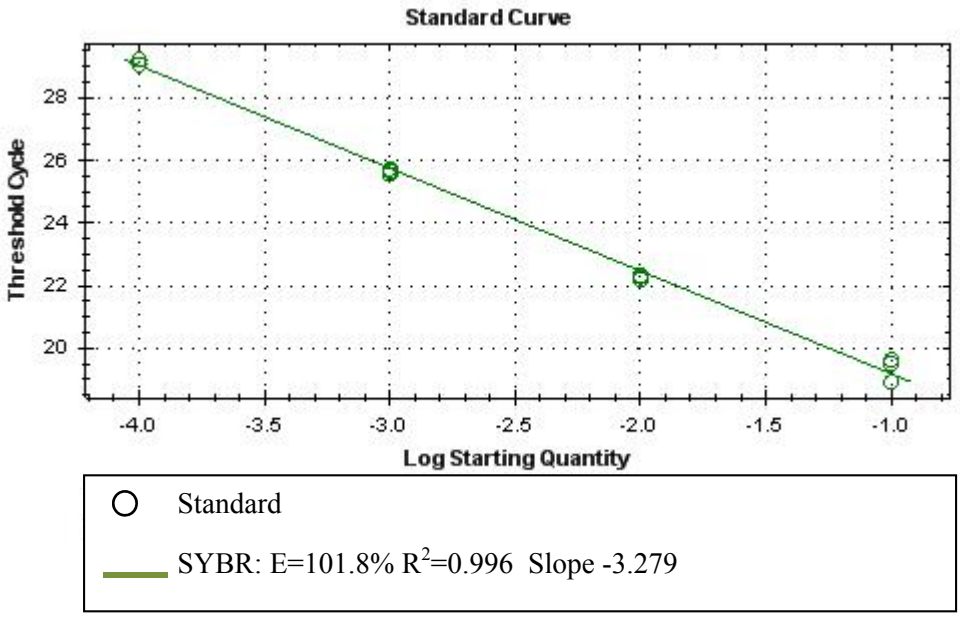

Figure 11. Standardization of primers for the *VbIAciBau3967_2681*gene.

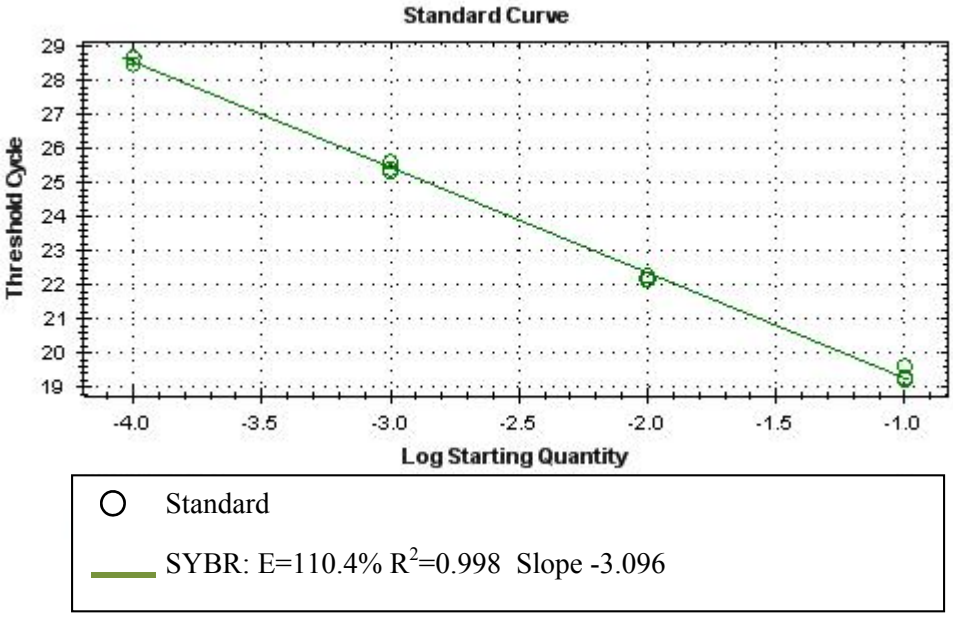

Figure 12. Standardization of primers for the *VbIAciBau3967_2424* gene.

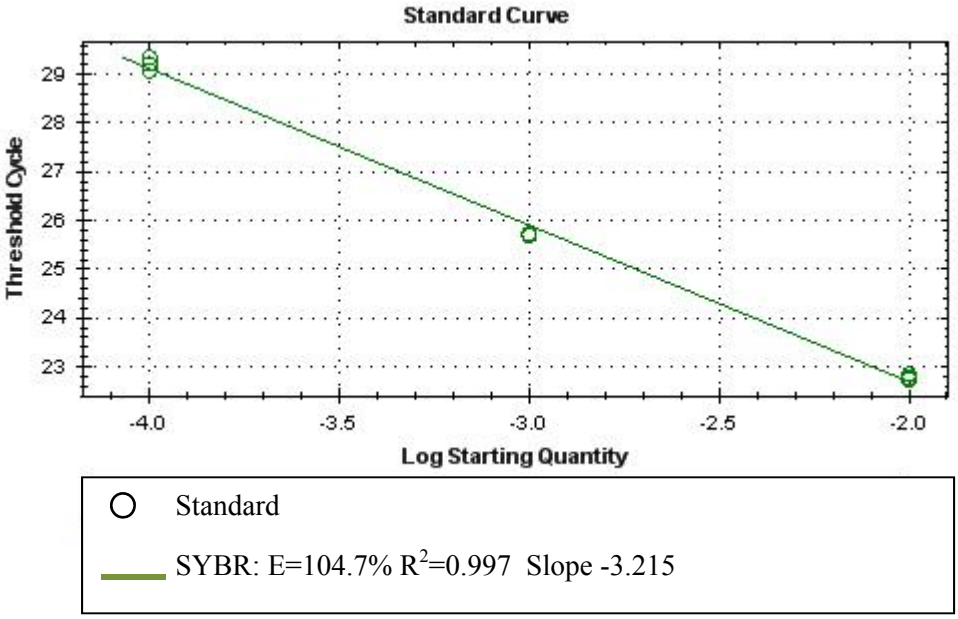

Figure 13. Standardization of primers for the *VbIAciBau3967_3362* gene.

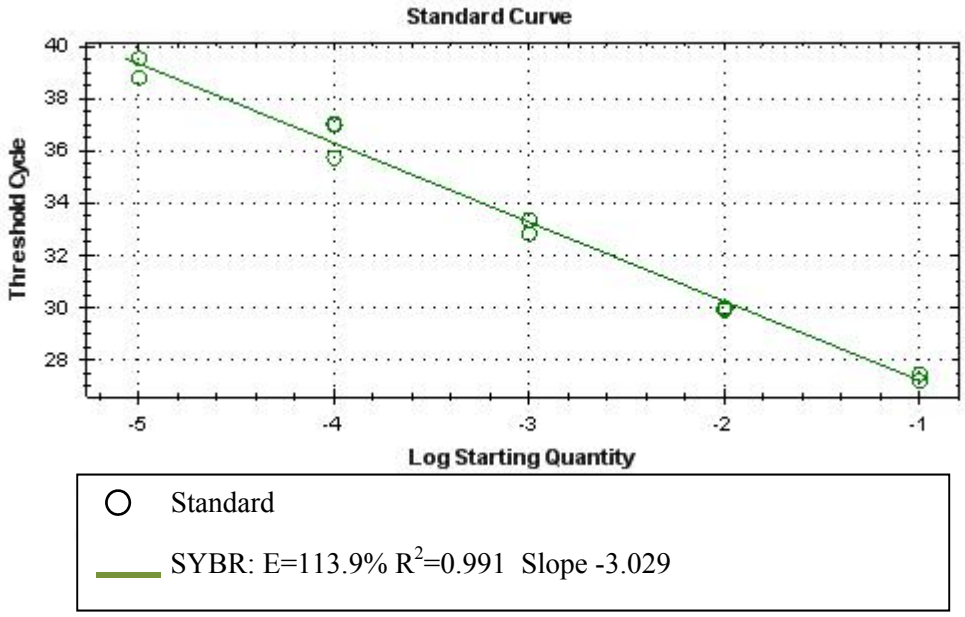

Figure 14. Standardization of primers for the *bfmR* gene.

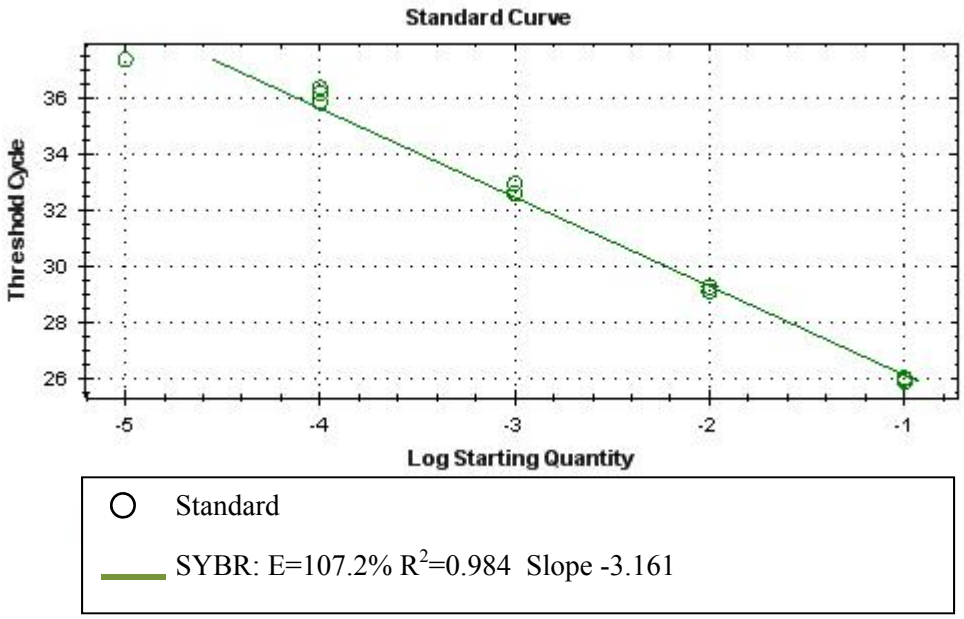

Figure 15. Standardization of primers for the *fmcC* gene.

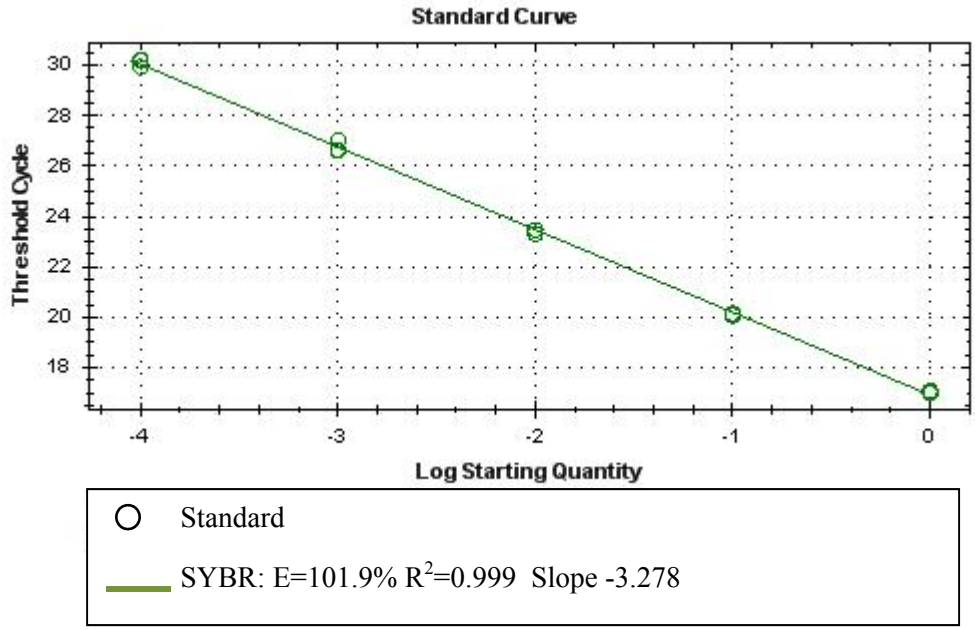

Figure 16. Standardization of primers for the *adeR* gene.

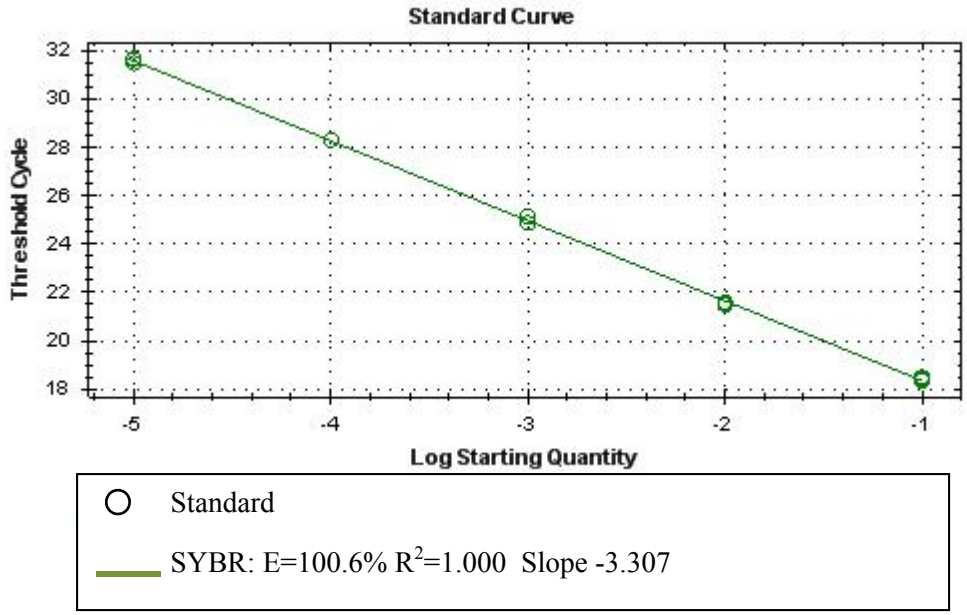

Figure 17. Standardization of primers for the *hsp90* gene.

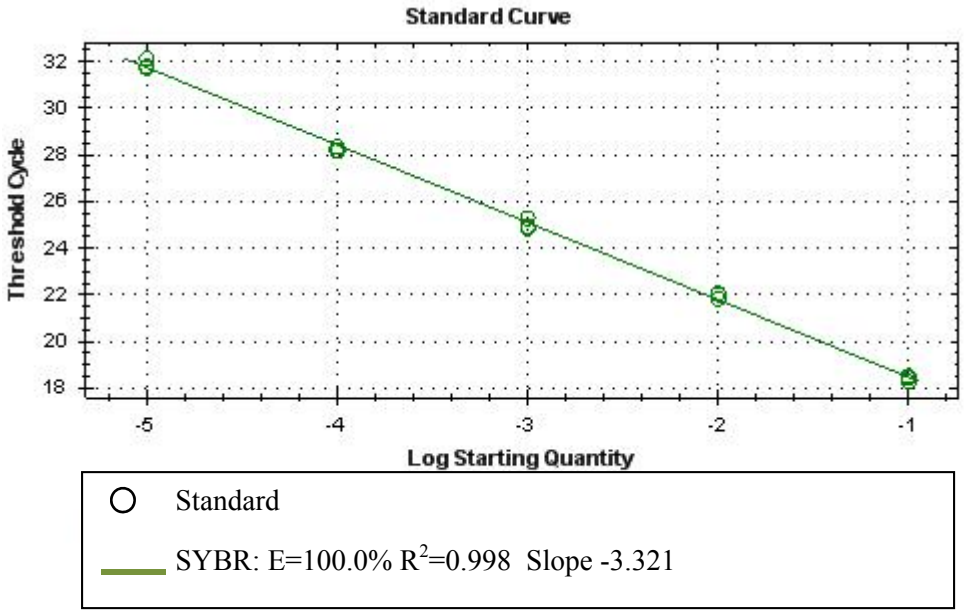

Figure 18. Standardization of primers for the *groEL* gene.