Construction and development of bioluminescent *Pseudomonas aeruginosa* strains; application in biosensors for preservative efficacy testing

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Submitted to the University of Hertfordshire in partial fulfilment of the requirements of the degree of Doctor of Philosophy

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(January 2014)

Dedication

In loving memory

To my late father Chimanlal Meghji Shah,

I dedicate this thesis to you.

Your words of encouragement and achievement have brought me to witness life to this date

and rest assured that your teachings and guidance

will always walk me through the right path in life.

Your inspiration and unconditional love and support

in pursuit of excellence will always live on.

Acknowledgements

This study was conducted at the University of Hertfordshire in partial fulfilment of the requirements of the degree of PhD under the supervision of Dr. David Naseby and Dr. Andreas Kukol.

First and foremost, I would like to thank my greatest teacher of all: God, the reason that I have reached this far and able to pursue this PhD. I will do my best in never forgetting what a great fortune I have had in just being here, and that it comes with a lesson and a responsibility. I hope I am doing the work you have planned for me to do.

I am utterly grateful to the University of Hertfordshire for awarding me with a studentship; this PhD would not have been possible otherwise. The financial award allowed me to obtain my education and further contribute to science in the field of biotechnology.

My sincere thanks and appreciation to Dr. David Naseby, my research supervisor who not only chose this topic for study but also nurtured its successful realization through the very useful criticisms and materials provided during the research. Special thanks to Dr. Andreas Kukol, my second supervisor, for your support, advice and assistance throughout my research. My appreciation also goes to the academic and technical staff in the school of Life and Medical Sciences for helping me through my research. Without you all, I would not have been able to conduct all those numerous experiments.

Many thanks to all my friends and colleagues that I have met in this my home far away from home called Kenya. Too many to name, but I would like to thank you all for your support and encouragement, sticking with me through the ups and downs.

Finally, I owe a great deal to my parents: Lila Chimanlal Meghji Shah and the Late Chimanlal Meghji Naya Shah. They gave me my name, they gave me my life, and everything else in between. They have been a constant source of support and encouragement and have made an infinite number of sacrifices for the entire family, and specifically for me to continue my schooling. They are a great inspiration to me, for without their understanding, I am sure this thesis would never have been completed. I pride myself in having words for everything, but no words can describe how much I love them and appreciate the efforts they have put into giving me the life I have now. They are the reason I did this; they are the reason I strive to be better. Their pride for me is my main goal in life. To my siblings, I thank you for your support and sacrifice.

Thank you, Thank you, Thank you

Declaration by candidate

I hereby declare that this thesis is a presentation of my original research work conducted at the School of Life and Medical Sciences at the University of Hertfordshire under the supervision of Dr. David Naseby and Dr. Andreas Kukol.

I certify that the research described is original and, wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

I also declare that I have written all the text herein and have clearly cited all references that have already appeared in publications.

Signature: Date:

Abstract

Whole cell biosensors have been extensively used for monitoring toxicity and contamination of compounds in environmental biology and microbial ecology. However, their application in the pharmaceutical and cosmetics industries for preservative efficacy testing (PET) has been limited. According to several pharmacopoeias, preservatives should be tested for microbial activity using traditional viable count techniques; the use of whole cell microbial biosensors potentially provides an alternative, fast, and efficient method. The aim of the study was to construct and develop whole cell microbial biosensors with *Pseudomonas aeruginosa* ATCC 9027. Constitutive promoters: P_{lysS}, P_{spc}, P_{tat}, P_{lpp} and P_{ldcC} and the *lux*-cassette were inserted into plasmid pME4510 and transformed into *P. aeruginosa* ATCC 9027 cells to produce bioluminescent strains. Plasmids were found to be maintained stably (~50 copies per cell) throughout the growth and death cycle.

The novel bioluminescent strains were validated in accordance with the pharmacopoeia using bioluminescence detection and quantification followed by comparison with the traditional plate counting method. The bioluminescent method was found to be accurate, precise and equivalent at a range of 10^3 – 10^7 CFU/mL, as compared with plate counting. Recovery of bacterial cells was quantified using bioluminescence; this method proved to be accurate with percentage recoveries between 70-130% for all bioluminescent strains. The method was also more precise (relative standard deviation less than 15%) than the traditional plate counting method or the ATP bioluminescent method. Therefore, the bioluminescent constructs passed/exceeded pharmacopoeial specified criteria for range, limit of detection, accuracy, precision and equivalence.

Physiology of the validated bioluminescent strains was studied by assessing the growth and death patterns using constitutive gene expression linked with bacterial replication. Promoter strengths were evaluated at various stages of the growth and death pattern and related to promoter sequences. P_{lysS}, P_{tat} and P_{lpp} were relatively strong promoters whilst P_{ldcC} and P_{spc} were relatively weak promoters. Relative promoter strength decreased in the order of $P_{lpp}>P_{tat}>P_{lysS}>P_{ldcC}>P_{spc}$ during the exponential phase whilst P_{tat} was stronger than P_{lpp} during the stationary phase of growth. P_{lpp} had its highest level of expression during the exponential phase, while P_{tat} had relatively stable *lux* expression during the stationary phase. Correlations between relative bioluminescence and CFU at 24 hours were greater than 0.9 indicating a strong relationship for

all bioluminescent strains. Reduction in correlation coefficients to approximately 0.6 between relative bioluminescence and CFU and between relative fluorescence and CFU beyond 24 hours indicated that a certain proportion of cells were viable but non-culturable. Tat-pME-*lux* showed steady bioluminescence compared to CFU count (R>0.9) throughout 28 days of growth. Equivalence analysis showed no significant difference between the bioluminescence and plate count method throughout 28 days of growth for all five bioluminescent strains.

Applicability of these novel bioluminescent strains was evaluated for preservative efficacy tests (PET) using bacterial replication and bioluminescence as a measure of constitutive gene expression. PET using benzalkonium chloride and benzyl alcohol showed no significant difference between the bioluminescent method and the plate count method. Good correlations between bioluminescence, CFU count and fluorescence were obtained for benzalkonium chloride (BKC) concentrations (R>0.9) between 0.0003% and 0.0025% against strains lysR25, lppR4 and tatH5. Similarly, good correlations (R>0.9) between the three parameters were obtained for benzyl alcohol (BA) concentrations between 0.125% and 2% against strains lysR25, lppR4 and tatH5. The bioluminescent method and traditional plate counting method were equivalent for concentrations of BKC (0.0003 - 0.02%) and BA (0.25 - 2%) during preservative efficacy tests. These bioluminescent constructs therefore are good candidates for selection for preservative efficacy testing. The bioluminescent method and traditional plate counting method were also found to be equivalent for construct tatH5 at a concentration of 0.125% BA.

PET testing with BKC and BA showed that tatH5-pME*lux* (R>0.9) had consistently high correlation coefficients between CFU and relative bioluminescence. Together with the results from growth and death kinetics, where tatH5 showed the greatest constitutive expression, it can be concluded that *P. aeruginosa* ATCC 9027 tatH5-pME*lux* is the best construct for testing various antimicrobial agents. This study has shown that according to the pharmacopoeial requirements, the bioluminescent method is more accurate, precise and equivalent to the traditional plate counting method and therefore can be utilised instead of the traditional plate counting method for the purpose of preservative efficacy testing.

Table of Contents

List of Figures

List of Tables

Abbreviations

3-(N-morpholino) propanesulfonic acid	MOPS
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
Absorbance	A
Adenosine triphosphate	ATP
Aldehyde	RCHO
Aminoacyl tRNA synthetases	aaRSs
Aminoglycoside acetyltransferase	AAC
Aminoglycoside nucleotidyltransferase	ANT
Aminoglycoside phosphoryltransferase	APH
Antimicrobial Efficacy Test	AET
Autoinducer	AI
Base pair	bp
Benzalkonium chloride	BKC
Benzyl alcohol	BA
β-galactosidase	β-gal
Biosynthetic lysine decarboxylase	ldcC
Blue Fluorescent Protein	BFP
Bovine serum albumin	BSA
Colony forming units	CFU
Correlation coefficient	R2
Crossing point	Cp
Cyan Fluorescent Protein	CFP
Degradative lysine decarboxylases	CadA
Deoxyribonucleic acid	DNA
Deoxyribonucleoside triphosphate	dNTP
Downstream core element	DCE
Downstream promoter element	DPE
ECF AlgU	RpoE or s22
Effective concentration	EC50
Energy-dependent phase I	EDP-I
Enhanced GFPs	eGFPs

Escherichia coli ATCC 8739	*E. coli* ATCC 8739
Ethylenediaminetetraacetic acid	EDTA
Extracytoplasmic function	ECF's
Faraday	F
Fatty acid	RCOOH
Fatty acyl group	RCOX
Genomic DNA	gDNA
Green fluorescent protein	GFP
Growth advantage in stationary phase	GASP
Initiator	Inr
Inner membrane	IM
Light addressable potentiometric sensor	LAPS
Limit of detection	LOD
Limit of quantification	LOQ
Lipopolysaccharides	LPS
Localization of lipoproteins	Lol
Luria broth	LB
Lysyl-tRNA synthetase	LysRS
Magnesium chloride	MgCl2
Melting temperature	Tm
Messenger RNA	mRNA
Minimum inhibitory concentration	MIC
Motif ten element	MTE
Multiple cloning site	MCS
N-acyl homoserine lactones	AHLs
Nicotinamide adenine dinucleotide phosphate	NADP
Nucleoside triphosphate	NTP
Ohms	W
Outer membrane	OM
Outer membrane lipoprotein	lpp
Oxidised flavin mononucleotide	FMN
P. aeruginosa ATCC 9027 + ldcC pME-*lux*	ldcC pME-*lux*

P. aeruginosa ATCC 9027 + lpp R3 pME-*lux*	lpp R3 pME-*lux*
P. aeruginosa ATCC 9027 + lpp R4 pME-*lux*	lpp R4 pME-*lux*
P. aeruginosa ATCC 9027 + lys G25 pME-*lux*	lys G25 pME-*lux*
P. aeruginosa ATCC 9027 + lys R25 pME-*lux*	lys R25 pME-*lux*
P. aeruginosa ATCC 9027 + pME4510	pME4510
P. aeruginosa ATCC 9027 + pME-*lux*	pME-*lux*
P. aeruginosa ATCC 9027 + spc pME-*lux*	spc pME-*lux*
P. aeruginosa ATCC 9027 + tat H5 pME-*lux*	tat H5 pME-*lux*
P. aeruginosa ATCC 9027 + tat H9 pME-*lux*	tat H9 pME-*lux*
P. aeruginosa ATCC 9027 + tat H14 pME-*lux*	tat H14 pME-*lux*
Percentage relative standard deviation	%RSD
Photobacterium phosphoreum	*P. phosphoreum*
Photorhabdus luminescens	*P. luminescens*
Piezoelectric	PZ
Plasmid copy numbers	PCN
Polymerase chain reaction	PCR
Preservative efficacy test	PET
Promoter for ldcC	P_{ldcC}
Promoter for lpp	P_{lpp}
Promoter for lysS	P_{lysS}
Promoter for spc	P_{spc}
Promoter for tat	P_{tat}
Pseudomonas aeruginosa ATCC 9027	*P. aeruginosa* ATCC 9027
Quantitative real-time PCR	qPCR
Quantitative structure-activity relationship	QSAR
Quarternary ammonium compounds	QACs
Quorum-sensing	QS
Reduced flavin mononucleotide	$FMNH_2$
Reduced nicotinamide adenine dinucleotide phosphate	NADPH
Relative fluorescence unit	RFU
Resistance-nodulation-division	RND

Restriction endonucleases	RE
Ribonucleic acid	RNA
RNA polymerase II	RNAP
RpoD	σ70
RpoF	FliA or σ28
RpoH	σ32
RpoN	NtrA, σ54 or σN
RpoS	σS or σ38
Sigma factors	σ factors
Sodium chloride	NaCl
Sodium chloride, Tris, EDTA	STE
Spc ribosomal operon	spc
Standard deviation	SD
Stationary phase contact dependent inhibition	SCDI
Statistical significance	sig
Sucrose & magnesium chloride electroporation buffer	SMEB
Super optimal broth with catabolic repressor	SOC media
The International Organisation for Standardisation	ISO
Threshold cycle	Ct
Tranfer RNA	tRNA
Tris EDTA	TE
Tryptone soya agar	TSA
Tryptone soya broth	TSB
Twin-arginine translocase	tat
Ultra violet	UV
Viable but non-culturable states	VBNC
Vibrio fischeri	*V. fischeri*
Volts	V
Wild-type *Pseudomonas aeruginosa* ATCC 9027	WT
Yellow Fluorescent Protein	YFP

Chapter 1: General introduction

1.1 Antimicrobial preservatives

1.1.1 Pharmacopoieal requirements

Substances that inhibit or prevent microbial growth and protect medicinal products from antioxidants, degradation and contamination are known as antimicrobial preservatives (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008; Kramer, Suklje-Debeljak & Kmetec, 2008). These are normally used to increase the shelf life of products. Antimicrobial preservatives are usually added to medicinal products that do not have antimicrobial activity (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008). Failure to add these preservatives can lead to product contamination and spoilage and can be a hazard to patients. Preservatives are chemicals that are usually aggressive to microbial cells. Hence they are used to inhibit the proliferation of micro-organisms, which under normal conditions would grow and contaminate medicinal products (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008).

Water-based medicinal products, such as solutions, creams, emulsions, suspensions, injections and eye-drops are most prone to microbial contamination. Hence, microbial preservatives must have the relevant properties to combat and prevent this contamination. These properties include concentration, pH, chemical composition and physical properties, the presence or absence of interfering substances (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008; Kramer et al., 2008). Other factors such as storage temperature, interactions between preservative and storage containers and packing play a vital role in preservative efficacy and must be tested under the regulations set out in several pharmacopoeias (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008; United States Pharmacopoeia, 2008).

The efficacy of the preservative may be altered by the formulation itself. Active ingredients and inert ingredients may both affect the efficacy of antimicrobial agents; albeit in different ways (Krouse, 2005; Parshionikar

et al., 2009; Springthorpe & Sattar, 2005). Likewise, changes in temperature, pH and humidity can alter the efficacy of preservatives (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008; Kramer et al., 2008). Product storage conditions and length of storage affect the product efficacy (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008). Furthermore, diluted products are less efficacious than concentrated products. Different microorganisms have different thresholds for survival when exposed to antimicrobial products (Parshionikar et al., 2009; Springthorpe & Sattar, 2005). The effectiveness of the preservative in the finished product must be tested over a period of time. Such tests are normally referred to as Preservative Efficacy Tests (PET) or Antimicrobial Efficacy Tests (AET) (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008); in such tests the medicinal product containing the preservative in its final container is inoculated with certain microorganisms and incubated at a specified temperature. Samples are then tested to assess the number of viable organisms present at various intervals of time up to 28 days after inoculation (Lundov, Moesby, Zachariae, & Johansen, 2009). Various pharmacopoeias also specify the type of media and diluents to be used for microbial growth and enumeration.

It is of great importance that the method of application of microorganisms is consistent throughout the tests, since different methods of application of test microorganisms may result in different amounts of microorganisms applied; this may affect efficacy of the antimicrobial product (Parshionikar et al., 2009; Springthorpe & Sattar, 2005). Several pharmacopoeial standards recommend a minimum inoculum of 10^5-10^6 organisms/mL. The minimum concentration of microorganisms recommended by the European pharmacopoeia is, however, 10^4 organisms for PET tests. More importantly, the characteristics of the microorganisms affects the outcome of testing (Parshionikar et al., 2009). For instance, less resistant organisms or exponential phase organisms may make the product appear more efficacious than it actually is, and *vice versa*. Stationary phase

organisms and more resistant organisms such as organisms with biofilms, may make the product appear less effective.

A significant reduction in the number of viable microorganisms in tests signifies that the preservative is effective. The criteria may vary slightly between different pharmacopeias. However, the acceptance criteria for liquid formulations according to the European criteria (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008) are summarized in Table 1.1. According to the European Pharmacopeia, successful formulations must pass criteria B, although it is recommended that criteria A be achieved (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008). Criteria A requires a 2 $\log_{10}$ unit reduction in number of bacterial colony forming units (CFU) by 6 hours post inoculation and a further 1 $\log_{10}$ unit reduction by 24 hours with no recovery in CFU for 28 days. If formulations fail to satisfy criteria A, they must satisfy criteria B, which requires a 1 $\log_{10}$ unit reduction in bacterial CFU by 24 hours and further 2 $\log_{10}$ unit reduction by 7 days with no further increase in CFU for 28 days (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008).

Table 1.1: European pharmacopoeial criteria for bacterial and fungal microorganisms. (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008)

Type of microbe	**Criteria category**	**Log unit reductions**				
		6 hours	24 hours	7 days	14 days	28 days
Bacteria	A	2	3	-	-	NR
	B	-	1	3	-	NI
Fungi	A	-	-	2	-	NI
	B	-	-	-	1	NI

Number of $\log_{10}$ unit reductions required for preservatives to pass the pharmacopoeial criteria. NR: No Recovery, NI: No Increase

According to the various pharmacopoeias (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008), the test microorganisms that need to be inoculated and tested are *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404 (British Pharmacopoeia, 2008; European Pharmacopoeia, 2008). The US Pharmacopoeia also requires the inoculation of *Escherichia coli* ATCC 8739 in addition to these five micro-organisms (United States Pharmacopoeia, 2008). This research project focuses on *P. aeruginosa* ATCC 9027.

1.1.2 Preservative efficacy failures

Microbial contamination in pharmaceuticals, food and cosmetics has been a problem for several decades, partly due to the difficulty in detecting it. It is therefore imperative that testing protocols for preservative efficacy be improved, such that all microorganisms are detected in due time. *P. aeruginosa* has been one of the most widespread opportunistic microbes to have been detected in various cosmetics. In 2013, a study found *P. aeruginosa* contamination in unopened toothpaste (Tan, Tuysuz, & Otuk, 2013). This pathogen, known to cause microbial keratitis, was also detected in a study investigating various contact lens disinfection solutions (Amiri, Mohammadinia, Tabatabaee, Askarizadeh, & Behgozin, 2011), which therefore failed to comply with the microbiological requirements set by The International Organisation for Standardisation (ISO). The importance of 'rubbing and rinsing' contact lenses in their multipurpose contact lens solution was highlighted in an investigation where *P. aeruginosa* was able to thrive in solution when a specific step was omitted (Zhu et al., 2011). A fatal episode involving a *P. aeruginosa* outbreak was observed in immunocompromised patients in a haematology unit in Italy, when the source of infection was heavily contaminated triclosan soap dispensers (Lanini et al., 2011). *P. aeruginosa* was also detected in cough syrups in 2009, when one of the preservative systems employed for a

cough syrup failed to be effective (Khanfar, Khalil, & AbuJafal, 2009). Many cosmetic products across Europe were recalled between 2005 and 2008 due to contamination with *P. aeruginosa* or other bacterial species (Lundov & Zachariae, 2008). A study investigating an outbreak of disease caused by contaminated mouth swabs across hospitals in Norway found that it was linked to *P. aeruginosa* and identified the source of the outbreak as occurring at various points in the manufacturing process (Iversen et al., 2007). A study in 2003 reported *P. aeruginosa* contamination in bottle warmers and milk bank pasteurisers in a neonatal intensive care unit (Gras-Le Guen et al., 2003).

These are just a few examples that emphasize how detrimental and lethal to human life this pathogen can be. Numerous investigations have been done for several decades that highlight the urgent need for novel methods for testing the efficacy of preservative systems.

1.2. Biosensors

1.2.1 General introduction to biosensors

Protection of human and environmental resources against deleterious agents such as microbes, toxic chemicals, pollutants and warfare agents needs the development of ‘real-time’ devices that can monitor these agents. The method of efficacy testing described in the pharmacopeias relies upon the growth of microorganisms, which may take at least three days for bacteria and up to a week for fungi. Hence, obtaining results is time-consuming. Sampling is also laborious and only four or five time points can be measured. This obviously has an effect on the time of release of products and therefore a cost implication. There is also a requirement for skilful personnel able to operate under the time constraints that the pharmaceutical, food and cosmetics industries currently face (Kramer et al., 2008).

Traditional methods for detecting microbial contamination have included morphological analysis and growth analysis on different media under various conditions. Polymerase chain reaction was the next technology used (Sperveslage, Stackebrandt, Lembke, & Koch, 1996); it was very sensitive but the analysis was time-consuming. PCR is a very sensitive method used to amplify minute quantities of DNA and it can therefore detect bacterial cells. Various instruments have been used to detect minute concentrations of microbes in clinical samples, food and water, such as flow cytometry (Perez, Mascini, Tothill & Turner, 1998), impedimetry, chromatography, infrared and fluorescence spectroscopy and bioluminescence (Ivnitski, Abdel-Hamid, Atanasov & Wilkins, 1999). Although traditional monitoring devices and detectors are available, the selectivity, specificity and limit of detection of these devices and the time it takes to monitor harmful agents are highly compromised. Furthermore, the requirement for sample preparation before detection does not allow monitoring in ‘real-time’. Hence, biosensor technology has recently been developed to detect these agents. Biosensor technology circumvents

some of the problems faced using traditional methods and also allows for real-time monitoring of these harmful agents (Nivens et al., 2004).

A biosensor consists of a biological sensing component, a transducer (Checa, Zurbriggen, & Soncini, 2012) and a signal processing element. The biological sensing component is the sensing device that contains biological molecules, such as whole cells, antibodies or enzymes (Nezich, 2007), that recognize an analyte or a physical/chemical change. The sensor is linked to a transducer, which converts the biological response from the sensing component into a different type of signal that can be processed further. Once an analyte or a change is identified, the transducer is activated and the signal is quantitatively detected (Daunert et al., 2000; Nivens et al., 2004). Hence, a biological response is converted into an electrical signal that can be processed and interpreted (Nezich, 2007). The sensing component and the transducer can be combined into a single element, for example an enzyme that catalyses a light-producing reaction.

Biosensors can achieve high selectivity so that a specific compound or a group of similar compounds can be detected. A wide variety of toxic compounds can also be detected. Another property of some biosensors is that they are very sensitive and therefore able to detect very low concentrations of an analyte. Biosensors are first classified as direct detection biosensors or indirect detection biosensors, depending on whether they measure the target analyte in real time (direct) or a reaction product of a biochemical reaction (indirect) (Ivnitski et al., 1999). Biosensors are further classified into four categories, based on the way the signals are transduced: optical, electrochemical, mass and thermal biosensors (Ivnitski et al., 1999). Direct detection biosensors include optical biosensors, bioluminescence biosensors, piezoelectric biosensors and impedance biosensors, whereas indirect biosensors include fluorescence-labelled biosensors, metabolism-based biosensors and electrochemical biosensors.

Optical biosensors utilize optical transducers that detect changes in thickness or refractive index of cells that bind to immobilized receptors (Watts, Lowe, & Pollardknight, 1994). Bioluminescence biosensors take advantage of light-emitting products produced as a result of enzymatic reactions and are used to monitor whether bacterial cells are present and the physiological state of these cells (Prosser, Killham, Glover & Rattray, 1996). Piezoelectric (PZ) biosensors comprise an antibody-coated PZ sensor that is then immersed into a bacterial solution (Le, He, Jiang, Nie & Yao, 1995). Binding of bacterial cells to antibodies causes an increase in mass. A reduction in resonance frequency of the oscillations is then measured. Bacterial Impedance biosensors utilize the fact that impedance decreases during bacterial metabolism and this is calculated by measuring several different parameters, such as conductance, capacitance or resistance (Hadley & Senyk, 1975). Bacteria form charged products from uncharged reactants causing an alteration in conductance.

Fluorescent biosensors utilize labelled molecules that emit light that is detected by fluorescence microscopy; for example, fluorescently labelled immunoglobulins (Brayton, Tamplin, Huq & Colwell, 1987). Such labelled immunoglobulins target carbohydrates and proteins that are embedded in bacterial cell membranes and therefore their presence can be used to detect the presence of bacterial cells. Metabolism-based biosensors monitor the metabolism of bacterial cells by measuring components of the electron-transport chain (Takayama, Kurosaki & Ikeda, 1993). Some transducers for these biosensors also measure an electrochemical metabolite or the use of oxygen during respiration. Electrochemical biosensors use a light addressable potentiometric sensor (LAPS) that comprises phosphorus coated with silicon and an insulator (Gehring, Patterson & Tu, 1998). When the silicon is immersed in an immunoreaction solution, there is charge redistribution and the voltage potential difference is measured.

1.2.2 Whole cell biosensors

Since micro-organisms are ubiquitous and continuously synthesize complex biomolecules in response to environmental changes, they have been used as whole-cell microbial biosensors. The advantage of using whole-cell biosensors is that they are able to perform complex biological processes in a complex matrix. They are able to respond to many environmental factors and changes in a very short period of time and using multiple pathways and reactions. There is no need for exogenous reagents to produce signals since endogenous coenzymes, cofactors and co-substrates necessary for enzyme reaction are already present in the cells. Hence, multiple biological-recognition and signalling for biosensors are possible since reporter micro-organisms support multi-enzyme reactions. The signals are detected by receptor proteins, which may be present in the microbial whole-cell or genetically introduced into the cell (Figure 1.1) (Nivens et al., 2004). Traditional analytical methods give an indication about the total concentration of particular analytes while whole cell biosensors provide information on bioavailability of pollutants, making it potentially a new technology for environmental monitoring (Sinclair, Paton, Meharg & Killham, 1999). The versatility of biosensors comes from their ability to measure low concentrations of chemicals, ability to work in complex systems and to have quick response times (Sinclair et al., 1999).

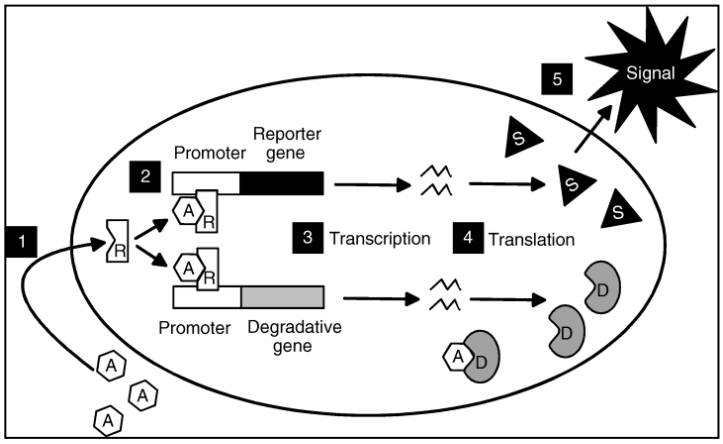


Figure 1.1: Gene expression of a bioreporter gene linked to a promoter. (Nivens et al., 2004) A: analyte, R: receptor, D: degradative enzyme as a result of degradative gene expression, S: signalling molecule. Under normal circumstances, A binds to R and the complex binds to the promoter to initiate expression of the degradative gene. In a biosensor, A being the autoinducer binds to its receptor R and the complex binds to the promoter that initiates transcription of the bioreporter gene. Expression of the reporter gene leads to production of the signal.

Cell-based bioreporters may require nutrition to survive and time to produce a detectable signal due to expression of reporter proteins and the transfer of analytes across the membrane, which limits the use of bioreporters (Nivens et al., 2004).

1.2.3 Applications of biosensors

Whole cell microbial biosensors can respond either to a specific analyte or generally to a group of similar analytes. Biosensors are powerful tools that are able to measure analytes at trace concentrations. The actual amount of analyte available to the biosensor can be measured rather than the total concentration. Depending on the specificity of biosensors, they can measure either a specific toxin or general toxicity of pollutants (Sinclair et al., 1999). Environmental contamination by heavy metals (Corbisier, Ji, Nuyts, Mergeay & Silver, 1993), organic compounds (Heitzer, Webb, Thonnard, & Sayler, 1992), industrial toxic waste (Brown et al., 1996), BTEX compounds (Sousa, Duffy, Weitz, Glover & Bar, 1998) and

chlorinated aromatics (Boyd et al., 1997) have all been detected by biosensors.

Phenolic compounds, such as chlorophenols, are widely used in preservatives, herbicides and fungicides and are toxic to microorganisms. Chlorophenols disturb the energy transduction pathway by either inhibiting electron transport or uncoupling oxidative phosphorylation. Bacterial biosensors containing *lux*-bioreporters depend on ATP, one of the products of the electron transport chain. Hence, disruption of the electron transport chain leads to inhibition of ATP production, which further reduces the light produced by the bioreporter (Sinclair et al., 1999). Such a system is an example where bacterial biosensors are used to assess chlorophenol toxicity.

Bacterial cells with a constitutively expressed reporter gene to monitor toxic compounds have been used as non-specific whole-cell biosensors (Hansen & Sorensen, 2001). The level of expression of the reporter gene is high in the absence of toxic agents; however, in the presence of the toxic compounds, the toxic effect on the bacteria leads to a reduction in light production, which can be measured. This is the basis of the commercially available Microtox® assay (Bulich, 1982). The reduction in metabolic activity may have been caused by changes in factors such as pH, salinity, presence of metals or xenobiotics; hence, the term non-specific whole-cell biosensors is used. The nature and severity of the actual toxic agent may therefore be difficult to assess with such types of biosensors (Hansen & Sorensen, 2001).

Stress-induced whole-cell biosensors have been used to study the effect of stress on bacterial cells. Stress may be induced by UV radiation (Vollmer, Belkin, Smulski, VanDyk & LaRossa, 1997), gamma irradiation (Min, Lee, Moon, LaRossa & Gu, 2000), antimicrobial agents (Bianchi & Baneyx, 1999) and oxidative stress (Belkin, Smulski, Vollmer, VanDyk & LaRossa, 1996). In these studies, stress-inducible promoters are linked to reporter genes to create stress-induced bioreporters. For example, the

promoter for *katG* gene is induced by stress caused by compounds such as cigarettes, alcohol and peroxides. The stress response usually involves a complex series of pathways and regulatory systems and is therefore non-specific and non-selective (Belkin et al., 1996). In another study of *Mycobacterium tuberculosis*, a pathogenic bacterium that causes tuberculosis, were exposed to low pH and oxidative stress and the heat-shock response was studied under these conditions (Wiles, Ferguson, Stefanidou, Young & Robertson, 2005). This was achieved by linking the *hsp60* promoter to the reporter gene. The light signal was obtained when the *hsp60* promoter was induced under the stressful conditions and the reporter gene was transcribed. Hence mycobacteria were able to survive under these stressful conditions.

Specific whole-cell biosensors have been used to study a specific response to different compounds (Hansen & Sorensen, 2001). In such studies, inducible promoters are linked to reporter genes and transcription of the reporter gene occurs when a repressor or activator protein sensitive to a specific molecule binds to the promoter (Hansen & Sorensen, 2001). For instance, xenobiotics such as phenols (Wise & Kuske, 2000), toluene-based compounds (Willardson et al., 1998) and middle chain alkanes (Sticher et al., 1997) induce specific promoters that regulate the genes for degrading these compounds. Hence, when these same promoters are linked to reporter genes, transcription of the reporter genes occurs, which subsequently gives an indication of toxicity caused by a specific compound. These xenobiotics are common soil pollutants and create a serious health risk if they are found in drinking water (Hansen & Sorensen, 2001).

1.2.4 The Microtox system

The Microtox system is a toxicity bioassay based on the natural bioluminescent bacteria *Photobacterium phosphoreum* and *Vibrio fischeri* (Nunes-Halldorson & Duran, 2003). It is available commercially and works

on the basis that a toxic agent alters the metabolism of these bacterial cells and eventually reduces cell viability. As the metabolism is reduced, the expression of the reporter gene is also reduced, resulting in a reduction in bioluminescence. The concentration of the toxic agent required to decrease bioluminescence by 50% is called the effective concentration (EC_{50}) and this value is used to measure the potency of the toxic compound. The Microtox assay is simple and highly sensitive, with a rapid response time. It can be used in Quantitative Structure-Activity Relationship studies, where the activity/efficacy of potential novel antimicrobial agents can be related to their structure. Such assays can also be automated, providing a platform for high through-put studies (Nunes-Halldorson & Duran, 2003).

1.2.5 Bioreporter genes

Several bioreporter systems in biosensing devices have been studied and utilized in research. These include the β-galactosidase system, green fluorescent protein system and luciferase system.

1.2.5.1 β-galactosidase

The simplest, most widely used and best studied reporter gene is the *lacZ* gene that codes for the enzyme β-galactosidase (Hansen & Sorensen, 2001). β-gal can be used to examine the transfection efficiency of plasmid and virus vectors (Yagi, 2007). β-gal converts a substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a colourless analogue of lactose, into a blue insoluble product 5-bromo-4-chloro-3-hydroxyindole and galactose. Hence, it can be detected by colorimetric analysis. It can also convert a range of other substrates into different coloured products. Specific inducible promoters linked to the *lacZ* reporter gene have been made and developed as bacterial biosensors, for example to detect xenobiotics and viruses. Biosensors using the *lacZ* gene have also been

utilized for the detection of toxic compounds (Ramanathan, Shi, Rosen & Daunert, 1998), chlorocatechols (Guan et al., 2000) and heavy metals (Klein, Altenbuchner & Mattes, 1997). Chemicals can be detected *in situ* and in real-time mode by performing electrochemical assays that utilize β-gal (Yagi, 2007). Although the *lacZ* gene can be expressed under most conditions, it requires no oxygen, ATP or cofactors for expression and can use a variety of substrates to produce coloured, easily detectable products. Its use, however, can be limited, since tedious protein extraction processes followed by β-galactosidase assays are required for accurate quantification of β-galactosidase (Hansen & Sorensen, 2001).

1.2.5.2 Green fluorescent protein (GFP)

The *gfp* gene, which encodes for green fluorescent protein, has also been widely used recently. GFP is an auto-fluorescence reporter protein that does not require ATP or any substrates (Roda, 2010; Yagi, 2007). The GFP protein requires light at wavelength 395nm or 488nm in order to fluoresce (Hansen & Sorensen, 2001). GFP as a bioreporter has been used to detect and quantify L-arabinose and also to discriminate between D-arabinose and L-arabinose (Shetty, Ramanathan, Badr, Wolford & Daunert, 1999). The GFP protein is a very stable protein molecule, although variants with a shorter half-life have also been produced (Hansen & Sorensen, 2001). Variants of GFP protein with half-lives as short as 40 minutes have been found to be useful for time-dependent induction studies. On the other hand, variants with half-lives greater than 24 hours have been used in biosensor studies, where time-dependent accumulation in cells or expression from weak promoters or expression under unfavourable metabolic conditions is being studied (Hansen & Sorensen, 2001). Development of mutant GFP proteins, termed enhanced GFPs (eGFPs) offered enhanced stability, different spectral properties and better signal intensities (Yagi, 2007). Enhanced GFPs have a wide range of applications, from measurement of gene expression, identification of transplanted cells to analysis of differentiation processes in eukaryotes.

Several variants that emit light of different wavelengths upon excitation have been engineered; BFP (Blue Fluorescent Protein) (Heim & Tsien, 1996), CFP (Cyan Fluorescent Protein) (Heim & Tsien, 1996) and YFP (Yellow Fluorescent Protein) (Wachter, Elsliger, Kallio, Hanson & Remington, 1998), depending on the colour of light they emit. The advantage of having such variants is that they can be used in combination to detect several analytes. However, the use of GFPs and their variants as biosensors is still limited, owing to the fact that there is a time delay between protein synthesis and maturation to produce an active fluorophore (Yagi, 2007). However, the sensitivity of fluorescence is remarkably good and a single bacterial cell expressing the GFP protein can be detected (Hansen & Sorensen, 2001). The use of GFP has its limitations, such as the requirement for expensive fluorometers and flow cytometers for fluorescence analysis. Since, the lower limit of detection for GFP is greater than that of other reporter genes, GFP is less sensitive then other reporter genes (Hansen & Sorensen, 2001).

1.2.5.3 Uroporphyrinogen III methyltransferase and DsRed

The *cobA* gene from *Propionibacterium freudenreichii* (Wildt & Deuschle, 1999) and the *dsRed* gene from coral *Discosoma* spp. (Matz, Fardkov, Labas, Savitsky & Zaraisky, 1999) are other reporter genes of interest. The enzyme encoded by *cobA* gene is uroporphyrinogen III methyltransferase, which causes accumulation of tri-methylated compounds. Excitation of these trimethylated compounds with blue or ultra-violet light causes these compounds to fluoresce and emit a strong red light (Wildt & Deuschle, 1999). The protein encoded by *dsRed* gene is called DsRed protein, which emits red light upon excitation (Matz et al., 1999). The half-life for DsRed can range from several hours to several days and therefore it is not used widely as a biosensor reporter. Both *cobA* and *dsRed* genes can be used together with GFP protein, since they are easily distinguishable (Eckert et al., 2005; Hansen & Sorensen, 2001).

1.2.5.4 Luciferases

The bioluminescent reaction is catalyzed by enzymes known as luciferases and the substrates for these enzymes are termed as luciferins. Luciferases also require molecular oxygen for their catalysis (Meighen, 1991). The firefly luciferase is a 62-kDa monomer that is expressed by a gene *luc.* The oxidation of benzothiazoylthiazole luciferin by the luciferase in the presence of ATP produces oxyluciferin and light (luminescence) (Wiles et al., 2005; Yagi, 2007). The luciferase from the jelly fish *Aequorea victoria*, called Aequorin, oxidizes the luciferin coelenterazine in the presence of calcium ions and molecular oxygen to produce coelenteramide, carbon dioxide and blue light. The blue light lasts only for 3 seconds (Lewis, Feltus, Ensor, Ramanathan & Daunert, 1998). Aequorin has been used widely as a bioreporter in biosensing studies and immunoassays (Lewis et al., 1998). The bacterial luciferases (*Lux*) from various bioluminescent bacteria oxidise reduced flavin mononucleotides and fatty aldehydes in the presence of oxygen to produce oxidised flavin nucleotide, fatty acids and blue-green light (Yagi, 2007). The bacterial luciferase from *Photorhabdus luminescens* will be utilised in this study.

1.2.5.4.1 *lux* CDABE cassette

Bacterial luciferases are encoded by several genes located in the *lux* operon. The *lux* operon consists of five structural genes *luxC, D, A, B* and *E* and two regulatory genes *luxR* and *luxI*. The sequence of each structural gene in the operon is as *luxCDABE*, which is common for all *lux*-operons*.* An additional *luxF* gene located between *luxB* and *luxE* genes is present in most of the *Photobacterium* species and absent in *Vibrio* and *Xenorhabdus lux* systems (Meighen, 1991).

The *luxA* and *luxB* genes encode the α and β catalytic subunits (Figure 1.2) of the flavin monooxygenase luciferase, respectively (Fisher, Raushel, Baldwin & Rayment, 1995; Geiselhart, Osgood & Holmes, 1991; Yagi, 2007). This heterodimer is ~80kDa (Wiles et al., 2005). The luciferase catalyzes a chemical reaction that produces light (Geiselhart et al., 1991; Sung & Lee, 2004). It is comprised of α and β subunits of ~40 kDa and ~35 kDa, respectively. Bacterial luciferases lack metals, prosthetic groups and non-amino acid residues. The α subunit has a substrate binding site and is responsible for light emission and rate of catalysis, while the β subunit is responsible for the interaction between these processes (Nunes-Halldorson & Duran, 2003).

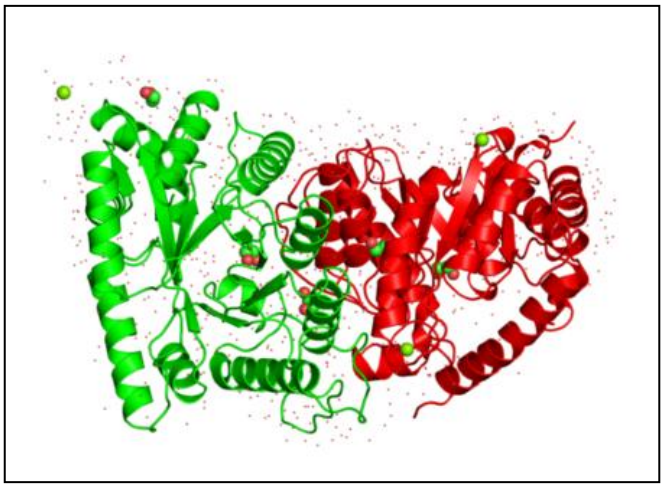


Figure 1.2: Three-dimensional structure of the luciferase heterodimer. The α and β subunits are shown in red and green respectively (European Bioinformatics Institute, 2008).

The *luxC, D* and *E* genes encode for the reductase, transferase and synthetase subunits of the multienzyme fatty acid reductase complex, respectively (Geiselhart et al., 1991; Yagi, 2007). The reductase component, also known as the acyl coenzyme A reductase, has a molecular mass of 54 kDa. The molecular masses of synthetase and transferase are 42 kDa and 33 kDa, respectively. The synthetase subunit is only weakly bound to the transferase subunit. Four reductase subunits

are found in the centre of the multienzyme complex and each reductase subunit is bound to the synthetase subunit. The total molecular mass of the multienzyme complex is therefore ~500 kDa (Wall & Meighen, 1986).

Most of the *Photobacteria* species have a flavoprotein in the *lux* system. The subunits of the flavoproteins have a molecular mass of approximately 24 kDa and are encoded by the *luxF* gene (O'Kane, Vervoort, Muller & Lee, 1987). The flavin prosthetic group of the flavoprotein consists of a flavin mononucleotide covalently linked to tetradecanoic acid. The function of this protein is unknown; however, it is not required for light production (Soly, Mancini, Ferri, Boylan & Meighen, 1988).

Two more genes, *luxG* and *luxH,* encoding for 25 kDa proteins, have also been discovered (Swartzman, Kapoor, Graham & Meighen, 1990). *Lux G* is known to encode for flavin reductase (Sung & Lee, 2004). *Lux H* is homologous to *RibB,* which encodes for DHBP synthase (O'Grady & Wimpee, 2008). This enzyme catalyses the reaction where ribulose 5'-phosphate is converted to DHBP (3,4-dihydroxy-2-butanone-4-phosphate) (Sung & Lee, 2004). This product undergoes further enzymatic reactions to generate riboflavin, which is used to generate FMN.

1.2.5.4.2 Mechanism of action of the *lux* cassette

The bacterial luciferase oxidizes a long chain aldehyde in the presence of reduced flavin mononucleotide to produce long chain fatty acid and blue-green light at 490nm (Nunes-Halldorson & Duran, 2003; Wiles et al., 2005). The firefly luciferase requires ATP, whereas the bacterial luciferase is highly specific for reduced flavin mononucleotide as the energy source. The overall bioluminescence reaction is:

$$O_2 + FMNH_2 + R\text{-}CHO \longrightarrow H_2O + FMN + R\text{-}COOH + light\ (\sim 495nm)$$

(Sung & Lee, 2004)

Binding of $FMNH_2$ to the luciferase enzyme E leads to the formation of an intermediate (EFH_2), constituting the first step of the luminescence reaction cascade (Nunes-Halldorson & Duran, 2003). Then EFH_2 reacts with molecular oxygen, leading to the formation of luciferase-bound flavin hydroperoxide intermediate (EFHOOH). Next, EFHOOH binds and reacts with the long chain aldehyde to form a flavin-oxygen-aldehyde-intermediate (E-FOOA). This particular step is the rate limiting step of the reaction in which an electron from the dihydroflavin molecule is transferred to the peroxide bond of E-FOOA, thereby exciting it and resulting in the breakage of the O-O bond (Nunes-Halldorson & Duran, 2003). The intermediate E-FOOA is very stable; its slow decay results in the release of energy in the form of light at 490nm (Meighen, 1991). Following excitation, the aldehyde is oxidized to form a long chain fatty acid, while oxidized flavin mononucleotide is released. The enzyme is then able to catalyze another reaction (Nunes-Halldorson & Duran, 2003). The enzyme flavin reductase, encoded by *lux F,* reduces the oxidized flavin mononucleotide (FMN) to form $FMNH_2$ (Sung & Lee, 2004). This reaction mechanism is depicted in Figure 1.3. The rate of turnover of luciferase in the bioluminescence reaction is less than the rate of chemical oxidation of $FMNH_2$; hence luciferases undergo only a single catalytic cycle (Hastings, Potrikus, Gupta, Kurfurst & Makemson, 1985). The fatty acid and reduced flavin mononucleotide are then available for another catalytic cycle.

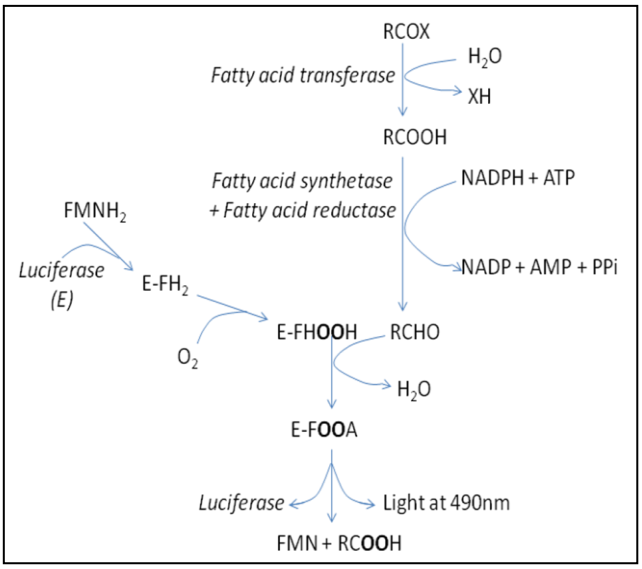


Figure 1.3: Reaction mechanism for bacterial *lux* enzymes (Adapted from Meighen, 1991). Catalysis of oxidation of aldehyde molecules into fatty acid molecules using energy and oxygen produces blue-green light measured at 490nm.

The multienzyme fatty acid reductase catalyzes the synthesis of aldehyde for bioluminescence reactions. The first step in the synthesis pathway is the transfer of fatty acyl groups to water or other oxygen and thiol acceptors. This step is catalysed by the transferase, which is also acylated during the reaction (Wall, Rodriguez & Meighen, 1986).

$$H_2O + RCOX \longrightarrow RCOOH + XH$$

Following the transfer of the fatty acyl groups to water, the fatty acid is activated with the aid of ATP by the synthetase component to form a fatty-acyl-intermediate. Once activated, the intermediate is reduced to aldehydes with the aid of NADPH. This reaction is catalyzed by the fatty acid reductase component of the multienzyme complex (Wall et al., 1986).

$$RCOOH + NADPH + ATP \longrightarrow RCHO + NADP + PPi$$

The light appears very dim in dilute cultures; however, the light intensity increases several thousand times during later stages of cellular growth.

The growth of bacterial cells therefore precedes the production and emission of light (Meighen, 1991).

1.2.6 Bioluminescence

Different types of signal output for bio-reporters exist; one of them is bioluminescence. The bioluminescence system is a highly sensitive and contained system for measuring cell viability and gene expression in real time (Wiles et al., 2005). Naturally occurring bioluminescent organisms are ubiquitous and well distributed throughout different species of fungi, fishes, insects and bacteria. Some species (for example, bacteria, algae, fireflies) have their own bioluminescent systems, whilst others (for example, fishes) have bioluminescent bacteria. The bioluminescence systems of luminous beetles (fireflies) and the luminous bacteria (*Vibrio* sp., *Photobacterium* sp. and *Photorhabdus* sp.) have been studied (Wiles et al., 2005). The luminous bacteria are the most widely distributed bioluminescent organisms and can be found in terrestrial environments and in water habitats. All the luminescent bacteria are classified into one of three genera, namely *Vibrio, Photobacterium* or *Xenorhabdus*; of them, *Xenorhabdus* infects only terrestrial organisms (Meighen, 1991; Robinson, Tonks, Thorn & Reynolds, 2011).

Free-living bioluminescent bacteria are found at dilute concentrations, whereas the bioluminescent bacteria living in the light organs of fishes and squid are found at very high concentrations of ~10^9 to 10^{10} cells per mL. The autoinducer concentrations increase to high levels in these organs to trigger light production (Zarubin, Belkin, Ionescu & Genin, 2012). The blue-green light produced is used to disguise the host animal from predators by counter-illumination (Dunn & Stabb, 2007). Luminous bacteria are attracted and ingested by predators such as fishes. These luminous bacteria, once ingested, reside and grow in the nutrient-rich gut environment (Zarubin et al., 2012). Hence, bioluminescence is used as a signal for food availability. Symbiotic relationships between bioluminescent

bacteria and squid have been described; the bioluminescence is used at night to mimic daylight so that light-dependent enzymes remain active for longer (Heath-Heckman et al., 2013).

The copepod *Guissia princeps* is a marine bioluminescent organism that produces a luminescent bolus in response to mechanical, electrical or light stimuli (Wiles et al., 2005). An example where a bioluminescent system acts as a line of defence is where the bolus blinds dark-adapted organisms while the copepod swims away from the predator.

Bioluminescent reporters have the potential to provide continuous real-time measurements of bacterial growth for application in the pharmaceutical, food and cosmetics industries. Therefore, failing formulations can be identified early, potentially saving much time and money. They can also be used as a rapid screening tools for the selection and development of formulations, antimicrobial products and preservatives (Naseby, 2007, 2008, 2009). Microorganisms are engineered to express the genes required for bioluminescence. The amount of bioluminescence is subsequently measured by a luminometer, which takes only minutes to measure the light intensity. A high bioluminescence reading indicates the presence of a large number of viable microbial cells and a low bioluminescence reading indicates the presence of a small number of viable microbial cells. In the presence of preservatives, the reduction in bioluminescence indicates a reduction in number of viable cells. Hence, such biosensors provide a remarkably useful tool for estimating microbial numbers at various stages of pharmaceutical manufacturing; in raw materials, semi-processed, processed and stored products.

1.2.6.1 Regulation of bioluminescence

The bioluminescence process, like any other process needs to be regulated. It is regulated by a phenomenon called autoinduction or quorum-sensing (QS) (Bassler & Losick, 2006). Initially observed in the bacteria *Vibrio fischeri,* autoinduction is a phenomenon in which bacterial

cells communicate with each other to assess the size of the bacterial cell population; this then results in the expression of certain proteins. Bacterial cells produce an extra-cellular signaling molecule known as an autoinducer (AI) (Nunes-Halldorson & Duran, 2003). N-acyl homoserine lactones (AHLs) are an example of autoinducers in gram negative bacteria (Miller & Bassler, 2001).

The regulatory mechanism is depicted in Figure 1.4. *LuxR* and *luxI*, two genes that are part of the *lux* operon, encode for an AHL receptor and an autoinducer AHL synthase, respectively, and are involved in the regulation of bioluminescence (Dunn & Stabb, 2007; Meighen, 1991). The *luxI* gene is transcribed in the opposite direction to that of *luxR* gene on the left operon. The AHL molecules may diffuse through the outer membrane into the culture medium or may be pumped out of the cell, depending on the length of the acyl side-chains (Kumari, Pasini & Daunert, 2008). Once in the culture medium, the AHL molecules bind to the AHL receptors; for example an AHL *N*-(3-oxo)-hexanoyl-DL-homoserine lactone (3-oxo-C6-HSL) in *V. fischeri* binds to its receptor LuxR, leading to the formation of a complex called AHL-LuxR (Kumari et al., 2008). This AHL-receptor complex subsequently binds to the promoter of the *lux* operon, P_{luxI} (Kumari et al., 2008), and stimulates transcription of the right operon (*luxCDABE*) (Dunn & Stabb, 2007; Meighen, 1991). Activation of the AHL-LuxR complex results in bioluminescence production. The gene *luxI* is also under the control of the same promoter and is therefore transcribed, leading to a positive-feedback loop mechanism (Kumari et al., 2008). Binding affinity of the receptor for AHL defines the concentration of autoinducer required to stimulate luminescence (Dunn & Stabb, 2007). Once a certain threshold level of AHL is reached, luminescence is produced by the cells (Nunes-Halldorson & Duran, 2003). The concentration of autoinducers is directly proportional to the bacterial load present in the environment (Bassler & Losick, 2006); hence, bacterial density can be determined by measuring luminescence intensity.

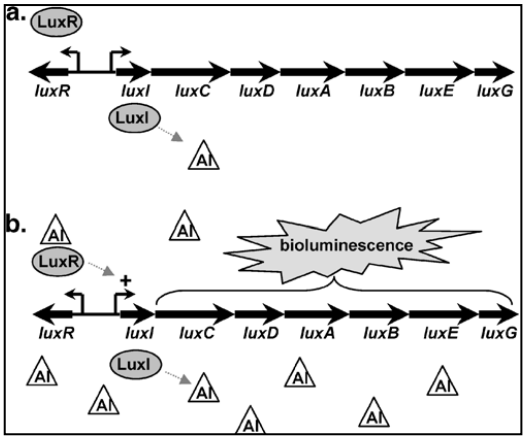


Figure 1.4: Autoinduction/Quorum sensing mechanism in *V. fischeri* (Dunn & Stabb, 2007). AHL synthase is constitutively expressed when cell density is low as depicted in (a). Synthesis of AHL by AHL synthase leads to the accumulation of AHL in the media. When bacterial cell concentration has reached a threshold (b), AHL binds to its receptor LuxR and the complex initiates transcription of the *lux*ICDABEG cassette (right operon) resulting in bioluminescence. Transcription of *luxI* also leads to more production of AHL synthase.

Bioluminescence by marine bacteria (Nealson, Platt & Hastings, 1970), biofilm formation in the lungs of cystic fibrosis patients by *Pseudomonas aeruginosa* (Middleton et al., 2002) and formation of virulence factors by *Staphylococcus aureus* (Miller & Bassler, 2001) are some of the characteristic responses produced as a result of QS. Various gastrointestinal (GI) tract illnesses due to pathogenesis are thought to be a result of QS (Kendall & Sperandio, 2007). In the case of bioluminescence, a minimum concentration of 10^7 cells/mL is required for the cells to produce luciferases and catalyze the bioluminescent reaction. Bacterial cells therefore assess their cell population in the environment and are able to cause an impact in the environment. Quorum-sensing is also used by bacterial cells to assess the population of other species in the environment (Nunes-Halldorson & Duran, 2003). Even though QS is beneficial with respect to bioluminescence, it is harmful with respect to pathogenesis and therefore there is a need to detect these autoinducers at an early stage. Whole cell biosensors in *Escherichia coli* and *Pseudomonas aeruginosa* have been developed for the detection of AHLs (Kumari et al., 2008).

1.3. *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous, opportunistic and extremely pathogenic gram negative bacterium found in the environment and it infects eukaryotic organisms such as mice, nematodes, insects, humans and also plants (Jander, Rahme & Ausubel, 2000). *P. aeruginosa* is known to infect patients who have undergone major surgery (Giamarellou, 2000), patients who suffer from cystic fibrosis, cancer, AIDS and patients who have severe burns (Lambert, 2002) or neutropenia (Bodey, Bolivar, Fainstein & Jadeja, 1983). *P. aeruginosa* isolates are known to produce biofilms around their cell membranes that provide a further protective barrier against any invasions. The bacterium produces a large number of virulence factors that provide nourishment for the bacterial cell and aid in the invasion of the host cell, causing damage to the host. These virulence factors interfere with eukaryotic signal transduction and metabolic pathways, resulting in the disruption of the cell, and hence they are toxic to eukaryotic cells (Nicas & Iglewski, 1985; Whitchurch et al., 2005). In cystic fibrosis, *P. aeruginosa* strains produce an alginate polysaccharide that surrounds the cells and aggregates them together (Lambert, 2002).

P. aeruginosa is still one of the main pathogens that is able to persist in harsh environmental conditions and in the presence of various antimicrobial agents (Abreu, Tavares, Borges, Mergulhao & Simoes, 2013). Attachement of *P. aeruginosa* to surfaces and production of biofilms thereafter has made this microorganism a critical problem in healthcare environments. Alcohols were employed traditionally as possible disinfectants. However, quaternary ammonium compounds have also been used recently to combat this pathogen (Abreu et al., 2013).

1.3.1 *Pseudomonas aeruginosa* susceptibility to aminoglycosides

Restricted uptake of drugs, drug inactivation and target alteration are the three basic mechanisms that enable bacterial cells to resist the action of antimicrobial targets (Lambert, 2002). Antibiotics used to treat infection caused by *P. aeruginosa* have to traverse the cell wall to reach their targets; however certain drugs have been shown to be effective. Protein synthesis in *P. aeruginosa* is inhibited by aminoglycosides, such as gentamicin, tobramycin and amikacin, that bind to the 30S subunit of ribosomes and cause misreading or suppression of protein synthesis (Brzezins, Benvenis, Weinstei, Daniels & Davies, 1972). The chromosome structure in cells is maintained by an enzyme called DNA gyrase, which is inhibited by quinolones such as ciprofloxacin. Transpeptidases that assemble peptidoglycan on the outer surface of the cytoplasmic membrane are inhibited by β-lactams, such as piperacillin, ceftazidime, imipenem. The membrane phospholipids that serve as a barrier to exogenous compounds are targeted by a class of antibiotics called polymixins, such as colomycin and colistin (Lambert, 2002).

A combined effect of restricted permeability of the outer membrane and efficient efflux of antibiotic molecules that manage to penetrate it are the two major factors that make *P. aeruginosa* resistant to a wide range of drug classes (Lambert, 2002). Hence drugs such as aminoglycosides and polymixins are used to overcome the restricted permeability barrier. When *P. aeruginosa* cells are exposed to aminoglycosides, a series of events occur that lead to the disruption of their cytoplasmic membranes. The potency of aminoglycosides towards micro-organisms depends on the number of amino-sugars linked to a cyclitol moiety (Mingeot-Leclercq, Glupczynski & Tulkens, 1999). Gentamicin binds to the lipopolysaccharides of the outer membrane, thereby making the cells more permeable (Lambert, 2002; Martin & Beveridge, 1986). After aminoglycoside binding, the outer membrane becomes more permeable

and therefore this facilitates further uptake of aminoglycoside molecules. This mechanism of uptake is called self-promoted uptake (Hancock, Raffle & Nicas, 1981).

After crossing the outer membrane, aminoglycosides traverse from the periplasmic space through the inner membrane in an energy-dependent manner termed energy-dependent phase I (EDP-I) (Bryan & Vandenelzen, 1975). This step of transporting aminoglycosides through the inner membrane is a rate limiting step and is inhibited by divalent cations, high acidity or low O_2 concentration (Xiong, Caillon, Drugeon, Potel & Baron, 1996). Observation under an electron microscope suggests that initial formation of small vesicles containing materials of the outer membrane, followed by the appearance of holes first in the outer membrane, peptidoglycan layer and then in the inner membrane, results in the release of membranous materials (Lambert, 2002). Aminoglycosides bind to the 30S ribosomal subunit in another energy-dependent manner termed EDP-II, once in the cytoplasm (Bryan & Kwan, 1983). This leads to disturbance in the elongation of polypeptide chains, leading to premature termination (Melancon, Tapprich & Brakiergingras, 1992). These abnormal proteins are inserted into the cell membrane, resulting in hyper-permeability and increased uptake of aminoglycoside molecules (Busse, Wostmann & Bakker, 1992). Even though gentamicin inhibits protein synthesis by binding to the 30S ribosomal subunit, it is also responsible for causing cell death by disrupting the cell membranes (Martin & Beveridge, 1986; Mingeot-Leclercq et al., 1999).

The susceptibility of *P. aeruginosa* cells to antibiotics is greatly diminished by calcium and magnesium ions at small concentrations (Martin & Beveridge, 1986; Zimelis & Jackson, 1973). This is because aminoglycosides compete for cationic binding sites of lipopolysaccharides on the cell surface (Abdelsayed, Gonzalez & Eagon, 1982). Aminoglycosides disrupt the membrane upon binding whereas small divalent cations stabilise the membrane (Mao, Warren, Lee, Mistry & Lomovskaya, 2001). EDTA and polymyxin are also known to cause

excessive membrane leakage by removing calcium and magnesium ions as well as lipopolysaccharides and proteins (Martin & Beveridge, 1986; Zimelis & Jackson, 1973). Polymixin B destabilises the outer membrane of *P. aeruginosa* cells in a similar fashion to gentamicin, by binding to cation binding sites of LPS molecules (Delcour, 2009). This destabilisation results in a self-promoted uptake of polymixin B into the periplasmic space. Further transport into the cytoplasm occurs through diffusion of polymixin B through the inner membrane (Delcour, 2009). This diffusion is mediated by the hydrophobic fatty acid tail of polymixin B.

1.3.2 Antibiotic resistance in *Pseudomonas aeruginosa*

It is difficult to control *P. aeruginosa* with antibiotics because of its ability to adapt easily in response to environmental changes, to grow on a wide range of substrates and its ability to acquire resistance against a wide range of antimicrobial agents. This is possible because *P. aeruginosa* has a large genome of 6.36Mbp that comprises 5567 genes (Lambert, 2002). Some of these genes encode for several multi-drug efflux pumps and cephalosporinases (Moir, Di, Opperman, Schweizer & Bowlin, 2007). Its ability to acquire other genes for resistance from other organisms via plasmids, transposons or bacteriophages also adds to the antibiotic resistance of *P. aeruginosa* (Brzezins et al., 1972).

A number of resistance mechanisms have been identified in *P. aeruginosa* populations with multidrug resistance. The most common mechanism is the presence of efflux pumps that pump out antimicrobial drugs from the cell. This prevents the antimicrobial drugs from accumulating and acting on target areas within the cell (Tenover, 2006). Several mechanisms of resistance can co-operate together to produce multiple aminoglycoside resistance in *P. aeruginosa* (MacLeod et al., 2000; Miller et al., 1997; Miller, Sabatelli, Naples, Hare & Shaw, 1995).

1.3.2.1 Aminoglycoside resistance by enzymatic inactivation

Drug resistance may also result from drug inactivation by enzymes. These enzymes can be either chromosome-encoded or plasmid-encoded (Poole, 2005; Poole, 2011).

Various enzymes are involved in the modification/inactivation of aminoglycosides in *P. aeruginosa*; for example, aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC) and aminoglycoside nucleotidyltransferase (ANT) (Azucena & Mobashery, 2001; Smith & Baker, 2002; Wright, 1999). These enzymes are involved, respectively, in the phosphorylation, acetylation and adenylation of aminoglycosides. Moreover, resistant *P. aeruginosa* strains have several genes for encoding these enzymes, thereby conferring broad-spectrum resistance (Miller et al., 1995). The aminoglycoside gentamicin undergoes N-acetylation by the AAC group of enzymes.

1.3.2.2 Impermeability resistance

Compared to other *Enterobacteriaceae,* there is limited permeability through the outer membrane of *P. aeruginosa* (Price & Godfrey, 1974); this gives it resistance to a variety of drugs (Delcour, 2009; Moir et al., 2007; Poole, 2005). This mechanism of resistance includes resistance acquired as a result of reduced uptake and therefore reduced accumulation of aminoglycosides in the cell of *P. aeruginosa* (Bryan, Haraphongse & Vandenelzen, 1976).

P. aeruginosa also lacks general diffusion porins, making the outer membrane less permeable. However, nutrients are transported through the outer membrane via specific porins (Nikaido, 2003). Many organisms including *E. coli*, *P. aeruginosa*, *Neisseria gonorrhoeae*, *Enterobacter aerogenes* and *Klebsiella pneumonia* (Achouak, Heulin & Pages, 2001; Hancock & Bell, 1988; Nikaido, 2003; Poole, 2004), have been shown to

acquire antimicrobial drug resistance via loss or mutations of porins (Goossens, 2003).

1.3.2.3 Acquired resistance

Although the outer membrane of *P. aeruginosa* provides a barrier against various compounds, high level multiple drug resistance is observed due to the presence of additional resistance mechanisms involving drug efflux systems (Ma, Cook, Hearst & Nikaido, 1994; Poole, Krebes, McNally & Neshat, 1993). The multidrug efflux pump also functions in synergy with the outer membrane impermeability mechanism (Germ, Yoshihara, Yoneyama & Nakae, 1999; Li, Zhang & Poole, 2000). Acquired multidrug resistance is usually caused by mutations leading to hyper-expression of efflux pump proteins (Kohler et al., 1996; Rella & Haas, 1982). These pumps normally transport dyes, detergents, inhibitors, disinfectants, organic solvents and homoserine lactones as well as antimicrobial agents (Poole, 2001).

The *P. aeruginosa* acquired resistance mechanism is characterised by a family of efflux pumps belonging to the resistance-nodulation-division (RND) family (Nikaido, 1998; Poole, 2004; Saier, Tam, Reizer & Reizer, 1994). The RND family is one of five families of efflux pumps involved in multiple drug resistance. The efflux pump proteins are highly homologous to those found in *Stenotrophomonas maltophilia, Burkholderia cepacia, Burkholderia pseudomallei* and the non-pathogen *Pseudomonas putida* (Poole, 2000).

An example of such an efflux pump in *P. aeruginosa* is the MexAB-OprM pump (Gotoh, Tsujimoto, Poole, Yamagishi & Nishino, 1995; Poole et al., 1993). This pump is expressed constitutively in wild type cells, providing intrinsic resistance. The *oprM* gene has been found to be conserved in all *P. aeruginosa* strains (Bianco, Neshat & Poole, 1997) and therefore may provide intrinsic resistance to all *P. aeruginosa* strains. This particular

pump may also be hyper-expressed in mutated strains (Kohler et al., 1996; Rella & Haas, 1982) and clinical isolates (Piddock, Hall, Bellido, Bains, & Hancock, 1992; Ziha-Zarifi, Llanes, Kohler, Pechere & Plesiat, 1999). The other efflux pumps are not detectable in wild type cells but are hyper-expressed in mutant *in-vitro* isolates and clinical isolates.

1.3.2.4 Adaptive resistance

Adaptive resistance (Houang & Greenwood, 1977) refers to the ability of *P. aeruginosa* to adapt itself and grow in the presence of aminoglycosides. This type of resistance is characterised by the presence of an inducible aminoglycoside drug efflux pump, which is induced in the presence of aminoglycosides, resulting in active efflux of the drug (Hocquet et al., 2003).

An example of adaptive resistance is illustrated by an outer membrane efflux protein OprF, which exists in abundance; albeit predominantly in a closed conformation and rarely in an open conformation (Bratu, Landman, Gupta & Quale, 2007). Hence, antimicrobial agents are unable to penetrate through the outer membrane, which acts as a barrier.

1.4. Gene expression

Gene expression comprises transcription and then translation (Pace, 1973). The formation of messenger RNA (mRNA) from template DNA by RNA polymerases is called transcription and it is regulated by various factors (Pace, 1973). All prokaryotic genes and operons are preceded by promoter regions. These regions are specific DNA sequences that are recognised by RNA polymerases for transcription initiation. The initiation of transcription entails various steps: firstly, location of promoter by RNA polymerase and reversible binding of RNA polymerase followed by RNA polymerase and DNA conformational changes; then nucleoside triphosphate (NTP) binding to the functional RNA polymerase-promoter complex (initiation complex) and initial phosphodiester bond synthesis; finally the movement of RNA polymerase from the promoter for elongation of mRNA to occur (DeHaseth, Zupancic & Record, 1998).

1.4.1 Promoter regions

The DNA region which regulates the correct initiation of transcription is called the core promoter (Juven-Gershon, Hsu, & Kadonaga, 2006; Juven-Gershon & Kadonaga, 2010). In *E. coli*, four promoter elements on the template DNA have been identified. They include two hexamer promoter regions located at positions -10 and -35 upstream to the transcription initiation site, the DNA spacer separating the -10 and -35 promoter regions and the UP element located between positions -40 and -60 upstream to the transcription initiation site (Figure 5) (DeHaseth et al., 1998). The -10 region is known as the melting module because this is where the DNA double helix is melted (Mishra & Chatterji, 1993). The -35 region is called the recognition module (Mishra & Chatterji, 1993) and there is a 17 $\pm$ 2 bp region in between the -10 and -35 regions called the spacer region (Mishra & Chatterji, 1993). This spacer region is vital in transcription because it separates the melting and recognition modules enough for RNA polymerase to interact with the promoters effectively (Figure 1.5).

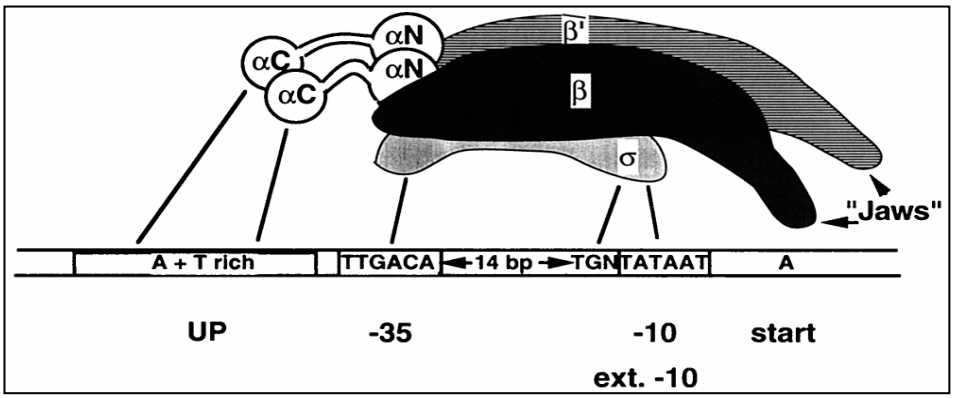


Figure 1.5: Model structure of RNA polymerase II interaction with promoter regions (DeHaseth et al., 1998). The consensus sequences for -10 and -35 regions are indicated. The UP element is A+T rich and upstream of -35 region. The TGN sequence forms part of the extended -10 region and is functional even in the absence of the -35 region. The 'jaws' is the catalytic site for RNA polymerase; makes contact with the DNA start site and initiates transcription.

Another conserved feature of promoters, apart from their sequence, is the flexibility of the DNA in that region (Vologodskii & Frank-Kamenetskii, 2013; Werel, Schickor, & Heumann, 1991). DNA bending occurs due to thermal fluctuations or protein binding (Huo et al., 2006). A poly-A tract found upstream of the recognition module creates a 'bend' in the local topology of DNA, thereby facilitating promoter recognition and binding (Travers, 1987). RNA polymerase recognises promoters by promoter sequence recognition and conformational changes in the promoter region by transcription factors (Tang, Deshpande & Patel, 2011). The promoter bending allows RNA polymerase to bind at promoter regions, while preventing it from binding non-promoter regions. They also allow RNA polymerase to scan for specific sites within the promoter regions to facilitate binding at the required promoter sequences (Tang et al., 2011). Promoter activity with respect to DNA topology has been studied previously (Huo et al., 2006; Mishra, Gopal, & Chatterji, 1990; Palecek, 1991) and can be classified into three types, depending on whether promoter activity increases, decreases or has no effect with respect to negative supercoiling of DNA. Up-regulation or down-regulation of gene expression has been shown to correlate with positioning of DNA bending

proteins (Huo et al., 2006). Hence, that variation in promoter activity is due not only to changes in promoter sequences but also to conformational changes in DNA topology and variations in supercoiling.

Promoter strength is defined as "the rate at which transcriptional elongation complexes leave a promoter thereby clearing the site for a subsequent encounter with RNAP" (Brunner & Bujard, 1987). It has been shown that RNA chain elongation occurs at a rate of 50 nucleotides/second (Brunner & Bujard, 1987) and therefore promoter clearance should take 1 second for effective promoter activity. Promoter strength increases as promoter sequences match the consensus sequences and therefore any alterations in promoter sequences lead to variations in promoter strength (Mishra & Chatterji, 1993).

1.4.2 RNA polymerase

RNA polymerase consists of four subunits β, β' and α_2 that form a heteroenzyme. The heteroenzyme forms an active holoenzyme upon binding of a σ factor (Figure 1.6) (DeHaseth et al., 1998). RNA polymerase holoenzyme bound to DNA occupies a region between -50 to +20 (Mishra & Chatterji, 1993). This region consists of the promoter regions that provide information about transcription efficiency.

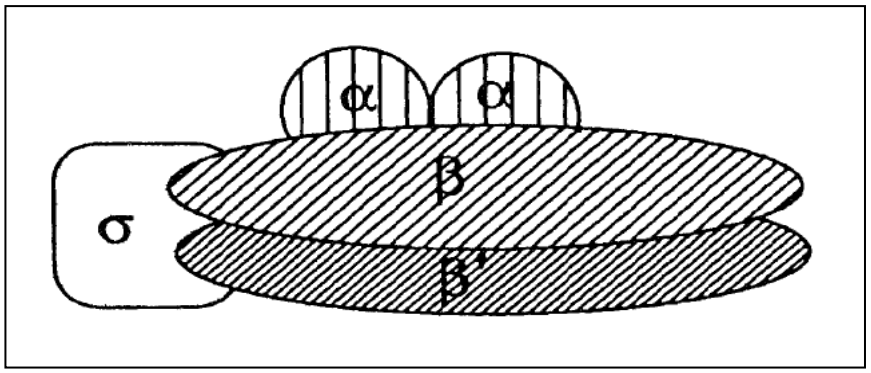


Figure 1.6. Subunits of RNA polymerase (Mishra & Chatterji, 1993). $\alpha_2\beta\beta'$ forms the RNA polymerase holoenzyme. Reversible binding of σ subunit forms the heteroenzyme.

The σ subunit recognizes the promoter even though it is weakly bound to the rest of the enzyme (the core polymerase, E). This subunit is eventually released after an initiation complex is formed. Then a phosphodiester bond is formed by the core polymerase, depending on the sequence on the template strand (DeHaseth et al., 1998). This process of NTP addition and phosphodiester bond formation to the elongating mRNA continues until the termination signal is reached. The ternary complex of core RNA polymerase, RNA and DNA dissociates and is released after chain termination. The core polymerase then binds to another σ subunit for the next transcription cycle (Travers & Burgess, 1969).

1.4.3 Sigma factors

The function of σ factors is to recognise promoter sequences. Various σ factors recognise specific promoter sequences and have different regulation properties (Barrios, Valderrama & Morett, 1999). *E. coli* has seven σ factors, whereas *P. aeruginosa* has 24 σ factors, of which 19 have extracytoplasmic functions (ECF's). The *P. aeruginosa* σ factors include RpoD (σ^{70}), RpoH (σ^{32}), RpoF (FliA or σ^{28}), RpoS (σ^{S} or σ^{38}), RpoN (NtrA, σ^{54} or σ^{N}); ECF AlgU (RpoE or σ^{22}), PvdS and SigX; and a collection of uncharacterized σ ECF. *E. coli* has σ^{70}, σ^{28}, σ^{32}, σ^{38}, σ^{54}, σ^{E} and σ^{FecI}; of which σ^{70} controls constitutive transcription during the exponential growth phase whilst the other factors regulate transcription during physiological and environmental changes (Blattner et al., 1997).

P. aeruginosa σ^{70} controls transcription of all housekeeping genes during the exponential phase of growth (Fujita, Tanaka, Takahashi & Amemura, 1994) and is highly homologous to *E. coli* σ^{70} (Tanaka & Takahashi, 1991). The -10/-35 promoter sequences are recognised by σ^{70} factors (Gruber & Gross, 2003; Thony & Hennecke, 1989).

The consensus sequences of the -10 and -35 promoter regions are TATAAT and TTGACC, respectively, in *E. coli*. The -10 region in *P.*

aeruginosa is also rich in A and T residues; however, the -35 regions may not conserved (Ramos & Marques, 1993). The *P. aeruginosa* -10 consensus sequence (TAtAAT) shows high similarity to the *E. coli* -10 consensus sequence (TATAAT). Similarly, the *P. aeruginosa* -35 consensus sequence (TTGACC) shows high similarity to the *E. coli* -35 consensus sequence (TTGaCc) (Potvin, Sanschagrin & Levesque, 2007). The greater the similarity of the -10 and -35 regions to their consensus sequence, the better the promoter function (DeHaseth et al., 1998). Spacer regions of 17bp ± 1 nt are required for functional σ^{70} dependent promoters; however, spacing regions between 15 and 20 nt have also resulted in effective transcription initiation (Ayers, Auble & Dehaseth, 1989; Hawley & McClure, 1983). σ^{70}-dependent promoters initiate transcription at a purine (Hawley & McClure, 1983). Although both -10 and -35 regions are vital for promoter activity, matching the -10 region to the consensus sequence is more important than matching the -35 region (Hawley & McClure, 1983; Lane & Darst, 2006; Rhodius & Mutalik, 2010).

Transcription may be further enhanced by transcription activator proteins binding to the α or σ^{70} subunits of RNA polymerase (Martin & Rosner, 2001; Vakulskas, Brutinel & Yahr, 2010). Promoters can be divided into two groups, depending on the location of the DNA sequence relative to the -35 region that is bound by the activator proteins in *P. aeruginosa* (Ebright, 1993). Hence, class one promoters have activator binding DNA regions 20bp upstream of the -35 region. Class two promoters have activator binding DNA sequences within or in close proximity to the -35 region.

P. aeruginosa σ^{54} is involved in flagellin biosynthesis (Dasgupta et al., 2003), pilin biosynthesis (Mattick, Whitchurch & Alm, 1996) and alginate production (Ma et al., 1998). *P. aeruginosa* σ^{54} has also been implicated in *P. aeruginosa* virulence (Hendrickson, Plotnikova, Mahajan-Miklos, Rahme & Ausubel, 2001). Promoters recognised by the E σ^{54} family are found in the -24 and -12 regions and contain conserved GG and GC dinucleotide motifs (Thony & Hennecke, 1989). The GG and GC motifs

found in the -24/-12 region specifically interact with the Eσ^{54} family (Cannon, Austin, Moore & Buck, 1995). A study showed that there are eight conserved nucleotides in the -24 region with the sequence mrNrYTGGCACG from -31 to -20 (Barrios et al., 1999) and similarly, there are 5 conserved nucleotides in the -12 region with the sequence TTGCWNNw from position -15 to -8. R represents a purine, Y represents a pyrimidine, W represents either A or T, m represents either A or C and N represents any nucleotide. They also show that a pyrimidine is always found at position -14, with T being the most common (Barrios et al., 1999). Similarly, a purine is most common at -20 whereas a pyrimidine is most common at -21. G is at least 96% conserved at positions -13, -24 and -25 whereas C is at least 96% conserved at position -12. Like σ^{70}- dependent promoters, σ^{54}-dependent promoters also initiate transcription at a purine (Hawley & McClure, 1983); however σ^{54}-dependent promoters become non-functional when deletion mutations occur in the -24/-12 regions (Keseler & Kaiser, 1995).

Other sigma factors include σ^{S}, σ^{38}, σ^{22} or SigX. RpoS or σ^{38} is involved in secretion of virulence factors like alginate (Hogardt, Roeder, Schreff, Eberl & Heesemann, 2004; Sonnleitner et al., 2003), regulation of quorum sensing (Latifi, Foglino, Tanaka, Williams & Lazdunski, 1996) and formation of biofilms (Davies et al., 1998) during the stationary phase of the growth cycle. *P. aeruginosa* σ^{S} is classified as a sub-group of σ^{70} and is involved in regulating transcription in response to environmental changes. These are involved in alginate biosynthesis and metal ion efflux (Raivio & Silhavy, 2001). It recognises the -35 promoter region with an AAC motif. *P. aeruginosa* σ^{22} is vital in converting non-mucoid cells to mucoid cells by interacting with promoter sequences responsible for transcribing alginates (Martin et al., 1993; Schurr, Yu, MartinezSalazar, Boucher & Deretic, 1996). *P. aeruginosa* SigX is also a sub group of σ^{70} and recognises the promoter sequence for transcription of OprF gene (Brinkman, Schoofs, Hancock & De Mot, 1999). The OprF protein is a membrane protein that forms part of the drug-efflux system.

1.4.4 Upstream elements

Regions downstream of -10 and upstream of -35 have been shown to be bound by RNA polymerase (Ozoline & Tsyganov, 1995). UP elements are not highly conserved; however, they consist of alternating A-tracts and T-tracts (Estrem, Gaal, Ross & Gourse, 1998) and are recognised by RNA polymerase bound with alternative σ-factors (Ross, Aiyar, Salomon & Gourse, 1998). UP elements are specifically bound to the α subunits of RNA polymerase (Landini & Volkert, 1995; Rhodius & Mutalik, 2010). UP elements are found between -40 and -60 in *E. coli* (Ramos & Marques, 1993). However, nucleotide sequences shorter than 20bp have also shown to function as UP elements. In some promoter regions, nucleotide sequences upstream of the -60 region have also contributed transcription initiation (Estrem et al., 1998). In *P. aeruginosa*, these regions lie between positions -40 and -80 (Ramos & Marques, 1993).

UP elements have been found to contain an 11bp distal region and a 4bp proximal region. The consensus sequence for the distal region is AAA(a/t)(a/t)T(a/t)TTTT, which appears between positions -57 to -47, whereas the consensus sequence for the proximal region is AAAA, which appears between positions -44 to -41 (Estrem et al., 1998). Although each region can function on its own, the regions from -51 to -53 and from -41 to -43 are vital (Estrem et al., 1998). Furthermore, the proximal region exerts a stronger effect on promoter strength than the distal region (Estrem et al., 1998). Strong promoters containing a proximal sequence further consist of A residues at positions -45 and -46 (Estrem et al., 1998).

1.4.5 Constitutive and induced gene expression

Initiation of transcription can occur in two different ways. Regulated genes usually consist of focused promoters that lead to focused initiation of transcription whereas constitutively expressed genes usually comprise of

constitutive promoters that lead to constitutive initiation of transcription (Figure 1.7) (Juven-Gershon, Hsu, & Kadonaga, 2006).

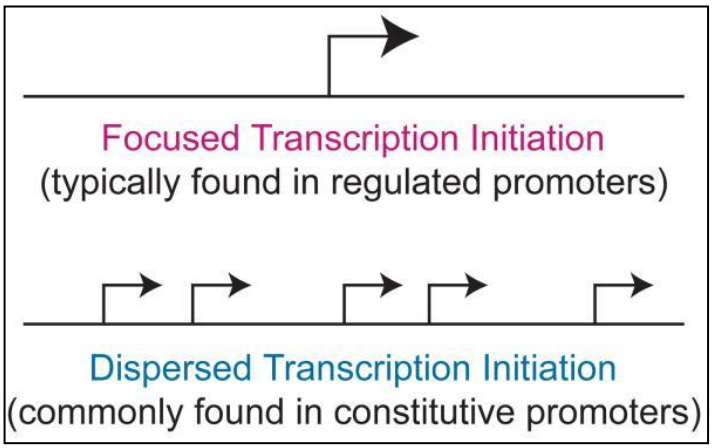


Figure 1.7: Modes of transcription initiation (Juven-Gershon et al., 2006). Focused mode of transcription initiation is found in regulated genes and consists of only a single major transcription start site. Dispersed mode of transcription initiation is found in constitutively expressed genes and consists of several weak transcription initiation sites.

Due to the existence of both constitutive and induced promoters, whole-cell microbial biosensors may be divided into two categories, based on the mode of transcription and therefore the type of signals they produce (Nezich, 2007). Micro-organisms that produce a signal constantly during a healthy state are classified as constitutive bio-reporters (Nezich, 2007). In constitutive bio-reporters, the signal production diminishes as cell metabolism is inhibited due to an increase in general toxicity level. Hence, these biosensors are used to measure general toxicity level in samples. Constitutive reporters have the benefit of producing a global response as a result of all the enzymes, cofactors and metabolic cascades present in the cell. They also have the benefit of assessing the general toxicity of the surrounding environment, thereby screening unexpected compounds as well. Micro-organisms that produce a signal when induced are classified as inducible bio-reporters (Nezich, 2007). These bio-reporters produce a signal only when their specific promoter is induced by its specific substrate, such as a heavy metal, pesticide, mutagen or organic solvent, and transcription is initiated.

Both types of bio-reporters can be used in biosensor technology, where the promoter is placed upstream to a reporter gene such as *lac Z*, *lux* or *luc* genes (Nezich, 2007). These genes produce either a colorimetric change or bioluminescence upon activation and the response can be monitored and quantified. Whole cell microbial biosensors produced for preservative efficacy testing in *E. coli* ATCC 8739 contain a constitutive promoter placed upstream of the *lux* cassette (Naseby, 2007, 2008, 2009). Five different constitutive promoters, namely P_{lysS}, P_{lpp}, P_{spc}, P_{tat} and P_{ldcC}, have been utilized to produce five biosensor strains of *E. coli* ATCC 8739 (Naseby, 2007, 2008, 2009). In the current study, the same constitutive promoters have been utilized. The genes associated with these constitutive promotors are now described in more detail.

1.4.5.1 Lysyl-tRNA synthetase (LysS) gene

Essential proteins like aminoacyl tRNA synthetases (aaRSs) are highly conserved proteins (Lee & Razin, 2005). Translation of mRNA into proteins is only possible if these enzymes can catalyze aminoacylation of tRNAs to form the 20 different aminoacyl-tRNAs required as building blocks for protein synthesis (Lee & Razin, 2005). One such enzyme, the Lysyl-tRNA synthetase (LysRS), catalyzes the aminoacylation of lysine to tRNA to form Lys-$tRNA^{Lys}$ (Jakubowski, 1999). There are two genes for lysyl-tRNA synthetase (LysRS); the constitutively expressed *lysS* gene and the inducible *lysU* gene (Emmerich & Hirshfield, 1987). The inducible gene *lysU* is induced by L-leucine, L-alanine, L-leucine dipeptides (Hirshfield, Yeh, & Sawyer, 1975), heat shock and growth at low pH (Hassani, Pincus, Bennett, & Hirshfield, 1992).

1.4.5.2 Spc ribosomal operon (spc)

Ribosomal proteins are encoded by genes that are arranged in operons (Cerretti, Dean, Davis, Bedwell & Nomura, 1983). Operons are found at

different loci in the chromosomes. One such locus, the *str* locus, comprises the *S10-spc-alpha* operons (Coenye & Vandamme, 2005) that encode for ribosomal proteins and are expressed constitutively. The *E. coli* spc operon consists of ribosomal genes *rplN, rplX, rplE, rpsN, rpsH, rplF, rplR, rpsE, rpmD* and *rpl10* that encode for L14, L24, L5, S14, S8, L6, L18, S5, L36 and L15, respectively (Cerretti et al., 1983). The spc promoter region is found between the gene encoding the S17 protein (last gene of the S10 operon) and the gene encoding the L14 protein (first gene of the spc operon) (Post, Arfsten, Reusser & Nomura, 1978). A substantial amount of transcription may start from the spc operon and continue into the α-operon downstream, thereby producing spc-α transcripts under normal growth conditions. This indicates that the expression of α-operon may be dictated by the spc promoter to a certain extent (Cerretti et al., 1983). Furthermore, the spc-α transcripts consist of several translation control points (Cerretti et al., 1983). Genes found in the *S10-spc-*α operon in *E. coli* are found outside these clusters in many genomes including that of *P. aeruginosa* (Coenye & Vandamme, 2005).

1.4.5.3 Outer membrane lipoprotein (lpp)

The inner membrane (IM) and outer membrane (OM) of gram-negative bacteria contain lipoproteins that not only maintain the structural integrity of cells but also mediate efflux of drugs and act as virulence factors (Nikaido, 1996; Remans, Vercammen, Bodilis & Cornelis, 2010b). Lipoproteins are synthesized as precursor proteins with an N-terminal signal sequence called the lipobox, which is responsible for the translocation of these proteins to the IM (Remans, Vercammen et al., 2010b). There are 90 and 185 different types of lipoproteins in *E. coli* and *P. aeruginosa*, respectively, and they are the most abundant membrane proteins (Remans, Vercammen et al., 2010b). Maturation of lipoproteins occurs at the IM, after which they are transported to the OM (Masuda, Matsuyama & Tokuda, 2002; Narita & Tokuda, 2007).

A mature lipoprotein consists of three acyl chains at the N-terminal that act as anchors in the membrane (Remans, Vercammen et al., 2010b). Transport of lipoproteins in bacteria is achieved by the Lol (*l*ocalization of *l*ipoproteins) proteins. There are five different Lol proteins that are expressed constitutively, LolABCDE (Narita & Tokuda, 2006; Tanaka, Narita & Tokuda, 2007; Tokuda & Matsuyama, 2004). The lipoproteins destined for the outer membrane are released from the inner membrane by the LolDCE complex (Remans, Pauwels et al., 2010a). A water soluble complex is formed between the lipoprotein and LolA, a molecular chaperone (Remans, Pauwels et al., 2010a), then crosses the periplasm to the OM and is received by the lipoprotein-specific outer membrane receptor LolB, a lipoprotein (Remans, Pauwels et al., 2010a).

Since the Lol components are crucial for bacterial cell growth, their reduction could therefore be fatal to bacterial cells (Narita, Tanaka, Matsuyama & Tokuda, 2002). Hence, the Lol components are targets for designing novel drugs aimed against pathogenic gram-negative bacteria. Recently, several compounds that inhibit the release of lipoproteins from LolA in *P. aeruginosa* have been developed (Pathania et al., 2009).

1.4.5.4 Twin-arginine translocase (tat)

Protein transport across the cytoplasmic membrane in bacteria is done by the sec-dependent and the twin-arginine-dependent pathways (Muller & Klosgen, 2005). The tat transporter has the ability to accept and transport folded proteins (de Keyzer, van der Does & Driessen, 2003; Muller & Klosgen, 2005). The tat pathway is vital in membrane development and cell division (Stanley, Findlay, Berks & Palmer, 2001), electron-transport chain and motility (Sargent, 2007), quorum sensing and pathogenesis (Ochsner, Snyder, Vasil & Vasil, 2002). Proteins transported by the tat pathway exhibit a consensus signal sequence S-R-R-x-F-L-K with x as a polar amino acid (Alami et al., 2003) in the N-region consisting of a twin-arginine motif (Berks, 1996). The tat transporter contains three proteins;

TatA, TatB and TatC (Muller & Klosgen, 2005) encoded by *tatA, tatB* and *tatC*, respectively (Sargent et al., 1998; Sargent, Stanley, Berks & Palmer, 1999). TatA promoter is responsible for constitutive expression of the tat genes (Jack, Sargent, Berks, Sawers & Palmer, 2001).

A homotetramer of TatB (Lee et al., 2006) and a homodimer of TatC (Punginelli et al., 2007) form a complex called TatBC signal recognition module and act as receptors for Tat substrates (Alami et al., 2003; Cline & Mori, 2001). Multiple protomers of TatA form the transport channel module (Dabney-Smith, Mori & Cline, 2006; Gohlke et al., 2005). Both modules exist as two separate entities under resting conditions (Dabney-Smith et al., 2006). During protein translocation, the TatC component of the signal-recognition complex recognizes the twin-arginine signal peptides (Alami et al., 2003; Cline & Mori, 2001), which bind to the TatBC complex resulting in a conformational change in the TatBC complex, leading to the exposure of the TatA-binding site (Cline & Mori, 2001). Binding of the signal peptide to the TatBC complex depends on the proton motive force (Gerard & Cline, 2007; Sargent, 2007). Then TatA binds to the substrate-bound TatBC complex (Alami et al., 2003) and the protein is translocated through the transport channel (Braun, Davis & Theg, 2007).

1.4.5.5 Lysine decarboxylase (ldcC)

Lysine decarboxylase is one of the many decarboxylases that attack basic amino acids (Lemonnier & Lane, 1998). Decarboxylases are classified into two categories. Degradative lysine decarboxylases (CadA) are induced by factors such as low oxygen tension, low pH and high concentration of their substrates (Lemonnier & Lane, 1998). Biosynthetic lysine decarboxylases (LdcC) are produced constitutively at low levels (Yamamoto, Miwa, Miyoshi, Furuyama & Ohmori, 1997) and catalyze the synthesis of polyamines (Krithika, Arunachalam, Priyanka & Indulekha, 2010; Tabor & Tabor, 1985). Expression of LdcC is very weak, suggesting that it is regulated by a weak promoter. Low concentrations of the enzyme suggest

that either transcription occurs at a low level or the mRNA transcripts are unstable (Lemonnier & Lane, 1998).

High concentrations of polyamines are found in gram-negative bacteria. Decarboxylation of L-lysine produces cadaverine, a precursor of polyamines. Expression of *cadA* (for lysine decarboxylase) (Meng & Bennett, 1992) is strongly induced under high acidity. To neutralize the pH, the reaction product cadaverine is released into the medium (Lemonnier & Lane, 1998). L-lysine decarboxylase requires the cofactor pyridoxal 5'-phosphate (Fothergill & Guest, 1977) and converts L-lysine to cadaverine (Qian, Xia & Lee, 2011) via the cadaverine or decarboxylase pathways (Figure 1.8). The ultimate product of this pathway is glutarate.

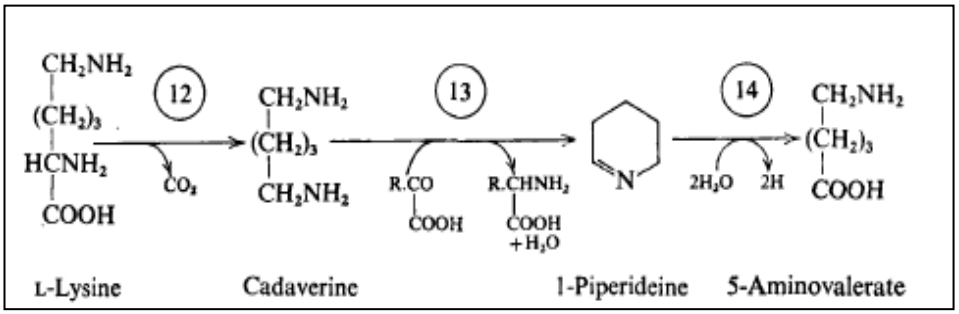


Figure 1.8: Cadaverine pathway (Fothergill & Guest, 1977). This pathway provides an alternative pathway for L-lysine metabolism. The first step of this pathway (Reaction 12) is the catabolism of L-lysine by lysine decarboxylase. Reactions 13 and 14 are catalyzed by cadaverine aminotransferase and I-piperidene dehydrogenase, respectively.

1.5. Rationale, aim and objectives of this project

Traditionaly preservative efficacy tests (PET) require inoculation of preservative formulations with specified micro-organisms, followed by plate counting techniques to monitor populations over the next 28 days. A further seven days are required for sample processing. Therefore the results are not usually available before 42 days after initial set-up. If formulations fail, repeated tests are required and new formulations may have to be designed. The traditional test is very laborious and hence not amenable to high throughput, rapid analysis. The development of biosensors for preservative efficacy testing will allow real time monitoring, rapid analysis and high throughput screening of preservative formulations, therefore saving time and labour inputs and allowing multiple formulations testing for rapid optimisation.

The overall aim of this project is therefore to develop novel whole cell *P. aeruginosa* ATCC 9027 biosensors. The main objectives of this study are:

- To construct whole cell *P. aeruginosa* ATCC 9027 bioluminescent constructs.
- To validate the bioluminescent constructs in accordance with several documents (ISO, 1994a, 1994b, 1994c, 2000; PDA, 2000), using the bioluminescent method and to assess plasmid copy number for all plasmid-bearing constructs.
- To determine the growth and death patterns of the bioluminescent constructs using the bioluminescent and plate count methods, to evaluate relative promoter strength for five pre-selected promoters and relate promoter strength to promoter sequence.
- To assess the applicability of *P. aeruginosa* ATCC 9027 and bioluminescent constructs for preservative efficacy tests, using benzalkonium chloride and benzyl alcohol.

Chapter 2: Genetic cloning

2.1 Introduction

Many applications of recombinant DNA technology have arisen since the advent of DNA cloning; for example, regulation of gene expression, creation of new proteins to study protein function, hormone synthesis for clinical and medical use, evolutionary studies of proteins, generation of pest resistant crops, to name just a few (Berg, Tymoczko & Stryer, 2002). DNA cloning is a widely used process in which DNA sequences from various different sources are joined together and replicated to produce larger amounts (Karp, 1999). This is done by introducing a foreign DNA segment into vector DNA and transferring the resulting recombinant DNA into competent host cells such as those of bacteria, fungi or eukaryotic cell lines. In this project, the *lux* cassette and promoters sequences will be joined into vector plasmids to create recombinant DNA, which will be transformed into *P. aeruginosa* ATCC 9027 to develop novel biosensors.

2.1.1 Plasmid vectors

Bacterial plasmids, used as vectors for DNA cloning, are small, circular, extra-chromosomal, double-stranded DNA molecules (Karp, 1999). These little entities have contributed significantly to the basic understanding of mechanisms in prokaryotic biology; these include gene expression, DNA replication and recombination (Khan, 1997). Plasmid vectors range in size from one kilobasepair (kb) to a few hundred kb (Antoine & Locht, 1992), consist of an origin of replication, some structural genes for antibiotic or heavy metal resistance (Lewin, 2008) and several restriction endonuclease sites, most of which are confined in the multiple cloning site (MCS). The vector plasmid pME4510 (Figure 2.1) from *Escherichia coli* DH5α cells (Rist & Kertesz, 1998) will be used in this study. The gentamicin resistance gene will allow for expression of proteins that confer resistance to gentamicin thereby providing a selective growth advantage

for host cells containing the plasmid, while inhibiting growth of cells that lack the resistance gene.

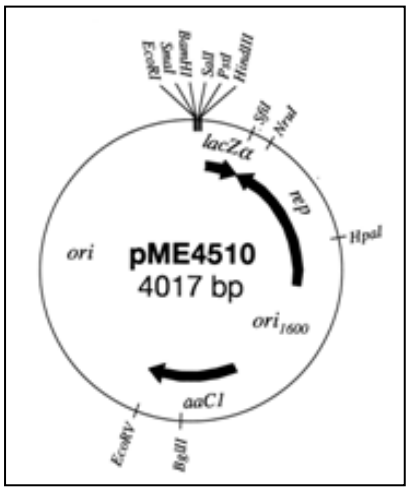


Figure 2.1: Plasmid map of pME4510 (Rist & Kertesz, 1998). The 4017bp plasmid contains a gentamicin resistance gene (aaC1), together with a number of restriction sites, some of which have been highlighted in the diagram.

Another feature of plasmids is their ability to be maintained through generations in a stable manner inside the host cells. Plasmids should be stable and free of burden on their host cell and should overcome segregational instability and competitive losses. Under certain conditions, plasmid encoded genes may offer a selective advantage to the host during growth and proliferation (Haines, Jones, Cheung & Thomas, 2005). For instance, the genus *Pseudomonas* carries many plasmids that offer environmental and clinical advantage to the bacterial hosts.

2.1.2 Recombinant DNA technology

One of the applications of recombinant DNA technology is the isolation of specific polypeptide-encoding genes (Karp, 1999). However, it may not always be feasible to isolate pure, good quality DNA in large enough quantities to be of any practical use in further downstream applications. Minute amounts of DNA are therefore amplified for such applications by polymerase chain reaction (PCR) (Mullis et al., 1986). The PCR procedure involves numerous cycles of DNA denaturation, primer hybridisation and polymerase extension, thereby 'amplifying' the DNA template. What makes this method so useful, is the fact that primers hybridise complementarily to their sequence on the DNA template, thereby making PCR highly specific, and the process doubles the concentration of DNA with each cycle making the fragment concentration increase exponentially (Mullis et al., 1986). Modification to primer design has allowed molecular biologists to directly clone DNA fragments generated by PCR by tagging restriction sites at one end of each primer (Kovalic, Kwak & Weisblum, 1991). As a result, a restriction site allows insertion of PCR products into DNA vectors upon digestion. Furthermore, directional cloning is possible by merely tagging each primer with a different restriction site.

Amplified DNA may be used further downstream for processes such as restriction digestion using restriction endonucleases (Pingoud & Jeltsch, 2001). The natural function of restriction endonucleases is to protect the host cells from invading foreign DNA, which could be detrimental to the host cell. The host cells are able to distinguish foreign DNA since the restriction sequences of host DNA are protected by methylation whereas foreign DNA is methylation-free (Pingoud & Jeltsch, 1997).

Foreign and host DNA produce complementary ends that can be linked together during the ligation process when they are digested with the same restriction endonucleases. The foreign DNA 'insert' ligates with the digested vector with the aid of an enzyme T4-DNA ligase, to form recombinant DNA (Karp, 1999). DNA ligase is an enzyme that catalyses

the formation of a phosphodiester bond between two adjacent nucleotides in the presence of magnesium ions and ATP (Montecucco, Lestingi, Pedralinoy, Spadari & Ciarrocchi, 1990a). Several DNA binding drugs, such as ethidium bromide, have been shown to interfere with the ligation reaction by intercalating into the minor grooves of DNA and thereby inhibiting the formation of active DNA-enzyme complexes (Montecucco, Pedralinoy, Spadari, Lestingi & Ciarrocchi, 1990b; Parolin et al., 1990).

2.1.3 Transformation

Recombinant DNA is introduced into host cells by a process known as transformation, after which the host's replication machinery replicates the DNA and passes it on to progeny cells called clones. Methods of delivering DNA into cells include bacterial conjugation, chemical transformation and electroporation. Transformation is an invaluable method in molecular biology, even though the procedures are very labour-intensive and time-consuming. Bacterial conjugation is the transfer of DNA from a donor to a recipient via direct contact between the cells and the recipient bacteria, often termed transconjugants (Woodall, 2003). For chemical transformation, the host cells are made competent by treating them with calcium ions (Karp, 1999). These cells then take up DNA when undergoing a heat-shock treatment. Electroporation is a technique that uses high voltage electrical pulses to create temporary pores in the cell membrane, thereby permeabilising the cell and subsequently introducing exogenous material such as proteins, DNA and RNA into the cell (Prasanna & Panda, 1997; Smith & Iglewski, 1989).

Some of the advantages of electroporation include greater transformation efficiencies and prevention of the use of toxic chemicals. The technique is fast, simple and reproducible with minimum expertise required (Prasanna & Panda, 1997). Although *E. coli* is fairly easy to transform, *Pseudomonas* cells have proven to be difficult to transfer. Part of the reason is because *Pseudomonas* cells produce high molecular weight polysaccharides called

alginates that form a layer around the cell membrane, which makes the cells viscous and hydrated and clumped together (Shen, Han, Lin & Yu, 2006). Numerous studies have utilised electroporation as the method of choice for transforming *Pseudomonas* only because other methods have very low transformation efficiencies and therefore a higher failure rate (Artiguenave, Vilagines & Danglot, 1997; Bassett & Janisiewicz, 2003; Diver, Bryan & Sokol, 1990; Farinha & Kropinski, 1990; Shen et al., 2006).

2.2 Aim and objectives

The aim of this work was to produce bioluminescent constructs of *Pseudomonas aeruginosa* ATCC 9027 using molecular cloning approaches. The objectives were:

- To isolate plasmid pSB417 (Figure 2.2) that contains the *lux* cassette from *E. coli* cells (Winson et al., 1998) and restriction to excise the *lux* cassette using restriction endonucleases.

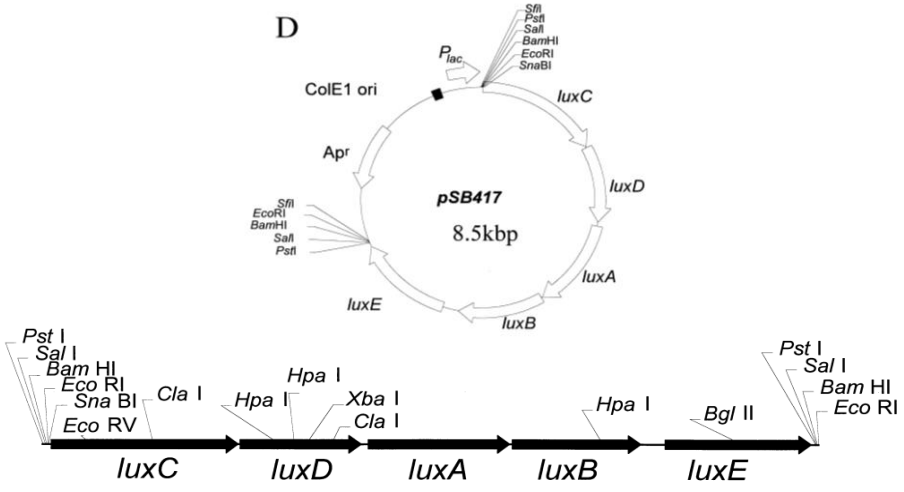


Figure 2.2: Plasmid map of pSB417 (Winson et al., 1998). The 8.5kbp plasmid comprises of the ampicillin resistance gene (Amp^r) and the *lux* cassette linked to the P_{lac} promoter. The *lux* cassette can be excised by a number of different restriction enzymes.

- To ligate the *lux* gene cassette into plasmid vector plasmid pME4510 to produce a promoterless plasmid pME-*lux*.

- To isolate genomic DNA from *P. aeruginosa*
- To amplify the promoter regions by polymerase chain reaction. The pre-selected promoters are as follows:
    - P_{lysS} Lysyl-tRNA synthetase promoter
    - P_{spc} Spc ribosomal protein operon promoter
    - P_{tat} Twin arginine translocase protein export system promoter
    - P_{lpp} Outer membrane lipoprotein promoter
    - P_{ldcC} Lysine decarboxylase promoter
- To digest the amplified promoter regions with restriction enzymes.
- To ligate the amplified promoter regions upstream of the *lux* gene cassette to produce recombinant plasmids.
- To transform recombinant plasmids into electro-competent *E. coli* cells.
- To screen transformants for recombinant plasmids containing promoter-*lux* inserts by ability to grow on selective media, PCR, bioluminescence measurements and DNA sequencing.
- To transform recombinant plasmids into electro-competent *P. aeruginosa* ATCC 9027 cells.
- To screen transformants for recombinant plasmids containing promoter-*lux* inserts by DNA sequencing, PCR and bioluminescence measurements.

At each stage, analysis will be done by agarose gel electrophoresis and/or absorbance spectrometry and/or luminometer.

2.3 Materials and methods

2.3.1 Bacterial strains

All bacterial strains used in this project are defined in Table 2.1.

Table 2.1: Bacterial strains used in this project

Bacterial strain	Source
Wild-type *E. coli* DH5α	Bethesda Research Laboratories., 1986
E. coli DH5α + pBR322	Balbas et al., 1986
E. coli DH5α + pSB417	Winson et al., 1998
E. coli DH5α + pME4510	Rist & Kertesz, 1998
E. coli DH5α + pBR322-*lux*	Provided by University of Hertfordshire
Wild-type *P. aeruginosa* ATCC 9027 (WT)	ATCC ®
P. aeruginosa ATCC 9027 + pME4510	Produced in this study
P. aeruginosa ATCC 9027 + pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + ldcC pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + spc pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + lys R25 pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + lys G25 pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + lpp R3 pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + lpp R4 pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + tat H5 pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + tat H9 pME-*lux*	Produced in this study
P. aeruginosa ATCC 9027 + tat H14 pME-*lux*	Produced in this study

2.3.2 Bioinformatics

Promoter sequences for the genes from *E. coli* were searched against the *P. aeruginosa* genome using BLAST to examine the similarity of these sequences in the two species. The five structural genes, namely ldcC, spc operon, tatABCD, lolB and lysS, in *P. aeruginosa* were then found in Genbank due to the fact that the promoter sequences from *E. coli* were very dissimilar in the two species. The sequences for the five structural genes, together with their upstream sequences, were used to search for the promoter sequences, using the software BPROM (Softberry, 2007) to identify the -10 and -35 positions. BPROM is a bacterial promotor prediction program with a reported accuracy of 80% in *E.coli*. Once the correct promoter sequences were identified, forward and reverse primers specific to each promoter were designed using PRIMER 3 software (Rozen & Skaletsky, 1998). The primers were designed in such a way that the resulting amplicons incorporated the -60 positions of the UP elements as well. Artificial restriction sites were also created at the 5' end of the forward primer and the 3' end of the reverse primer. The melting temperatures of the primers containing the restriction sites were calculated using 'BioMath - Tm Calculations for Oligos' software (Promega Corporation, Corporation). Webcutter 2.0 (Heiman, 1997) was used to ensure that the promoter sequences had no similarity with the artificially added restriction sites.

2.3.3 Gram staining and microscopy

P. aeruginosa was grown for 24 hours on a nutrient agar plate (Oxoid Ltd) incubated at 30^{o}C. The bacterial cells were prepared for gram staining according to the gram stain method (Gram, 1884).

2.3.4 Antibiotic testing

P. aeruginosa ATCC 9027 cells were inoculated from a single isolated colony into a culture flask containing LB broth (Oxoid Ltd) and incubated at 30^{o}C for 24 hours. Aliquots of the cell culture (100 μl) were then spread onto LB-agar (Oxoid Ltd) plates and incubated at 30^{o}C for 24 hours to create working plates. *P. aeruginosa* ATCC9027 cells were spread onto new LB plates from the working plate using an inoculation loop. Several antibiotic discs (Oxoid Ltd) were placed, in triplicate, with the aid of a disc dispenser onto the plates containing the *P. aeruginosa* cells. The antibiotics tested are shown in Table 2.2. The plates were incubated at 30^{o}C for 24 hours and examined for inhibition zones. Where present, the diameters of the zones of inhibition were measured using a ruler. The susceptibility of the cells to the antibiotic was then determined according to the BSAC standardised disc susceptibility testing method (Andrews, 2009).

Table 2.2: Various antibiotics for antibiotic resistance testing of P. aeruginosa*.*

Antibiotic	**Concentration (μg/disc)**
Polymixin B	300
Tetracycline	30
Trimethoprim	5
Erythromycin	15
Nalidixic acid	30
Gentamicin	10
Chloramphenicol	30
Streptomycin	10
Kanamycin	5
Vancomycin	30
Methicillin	5
Clindamycin	2

2.3.5 Genomic DNA extraction

P. aeruginosa was grown for 24 hours in a culture flask containing nutrient broth and incubated at 30^{o}C in a shaking incubator at 150rpm. Genomic DNA (gDNA) extraction from *P. aeruginosa* was done according to Cheng and Jiang (2006), after which the samples were purified and concentrated with alcohol. Following final incubation with chloroform, the upper aqueous phase (150 μL) was transferred to a sterile eppendorf tube and 15 μL of 3M sodium acetate was added. Cold absolute alcohol (375 μL) was added to the mixture, mixed gently and left for 24 hours to precipitate the DNA. The mixture was then centrifuged for 2 minutes. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol before centrifuging for 2 minutes. The pellet was dried for 5 minutes using speed vacuum to remove any residual ethanol. The resultant DNA pellet was subsequently resuspended in TE buffer (pH 8) and stored at -20^{o}C. The concentration and purity of DNA was determined by measuring the absorbance at 230, 260, 280 and 320nm on the Eppendorf spectrometer (Eppendorf®).

2.3.6 Polymerase Chain Reaction (PCR)

The promoter regions for each of the five genes, lysyl-tRNA synthetase (P_{lysS}), ribosomal operon (P_{spc}), tatABCD (P_{tat}), lysine decarboxylase (P_{ldcC}) and lipoprotein lolB (P_{lpp}) were amplified by designing specific forward and reverse oligonucleotide primers (Table 2.3). Oligonucleotide primers were constructed by Invitrogen Ltd. The primers selected to amplify the promoter regions were:

Table 2.3: Forward and reverse primers for amplification of five promoter regions P_{ldcC}, P_{lpp}, P_{lysS}, P_{spc} and P_{tat}.

Primer Name[a]	Primer	Primer length (bases)[b]	% GC content[c]	T_m (^oC)[d]
ldcC F	5'-CTTCAAGAATTCGGGTTACGCGCGTGCCGG-3'	30	60.0	67
ldcC R	5'-CTGATTACGTAGTCAAGAGAATGCTGAAGCCGTCCTG-3'	36	55.6	69
lpp F	5'-CTTCAAGAATTCGTATTGACCCCATAGACAGCTTCG-3'	36	44.4	64
lpp R	5'-GTGATTACGTAGTCAGCGCAAGGGATTTGTTCATA-3'	39	46.2	67
lysS F	5'-CTTCAAGAATTCGCTGTCTCTGGGAGCTACTCG-3'	33	51.5	66
lysS R	5'-GTGATTACGTAGTCAGATAGCGGCCGATTGATTC-3'	35	51.4	67
spc F	5'-CTTCAAGAATTCGTAACGCGCGGAACTTCCATC-3'	33	48.5	64
spc R	5'-CTCATTACGTAGTCAAGGTAGATCACCATGGCACGA-3'	36	52.8	68
tat F	5'-CTTCAAGAATTCGGGGTATTCCTGATCCTGCGCCG-3'	35	54.3	68
tat R	5'-GTGCATACGTAGTCAGGCGTTCCAGTGAATCTCATCCAG-3'	38	50.0	68

[a] F denotes forward primer and R denotes reverse primer. Underlined sequences show attached restriction sites. The primer length[b] is between 30-40bp, % GC content[c] is around 50% and the melting temperature[d] for each primer is between 60-70^{o}C.

Stock primer suspensions for tat, lpp, ldcC, lysS and spc were prepared by adding 100 μL of TE buffer pH 8. Working solutions of primers were prepared by adding appropriate volumes of TE buffer (pH 8) resulting in a concentration of 25 μM. The stocks and the working solutions were stored at -20^{o}C until required.

The initial PCR reaction mixtures were prepared in sterile PCR tubes (Table 2.4). Reaction buffer, magnesium chloride, deoxynucleotides, and Taq polymerase were purchased from Promega Ltd. The reagents were added, making up a total volume of 50 μL. Individual PCR reactions for each of the promoter regions were prepared.

Table 2.4: Various reagents for a PCR reaction.

Reagent	**Volume (μL)**[a]
10x Reaction buffer	5
25mM $MgCl_2$	3
10mM dNTP	1
25μM Forward primer	1
25μM Reverse primer	1
5U/μL Taq polymerase	0.2
DNA template	1
Sterile water	38.8

[a] Volumes of each reagent at stated concentrations required for 50 μL reaction.

The reaction tubes were placed in a Gradient PCR thermocycler (Eppendorf Mastercycler Gradient). The conditions used for the PCR reaction are summarised in Table 2.5.

Table 2.5: Cycling parameters for the PCR reactions.

Steps		Temperature (^{o}C)	Time (minutes)	
1	Initial Heat Denaturation	94	5	
2	Heat Denaturation	94	1	30 cycles
	Primer annealing	Gradient temperature	1	
	Primer extension	72	1	
3	Extension	72	10	
4	Quenching	4	infinite	

After optimizing the various reagent concentrations in the PCR reaction mixture and the cycling parameters, the final volumes of each reagent and the final annealing temperature for each of the five reactions were used (Table 2.6).

Table 2.6: Final reaction volumes and annealing temperatures for all five PCR reactions.

Reagent	Volumes (μL) for each prototype [a]				
	P_{tat}	P_{lpp}	P_{ldcC}	P_{lysS}	P_{spc}
10x Reaction buffer	10	10	10	10	10
25mM $MgCl_2$	4	4	3	6	6
10mM dNTP	2	2	2	2	2
25μM Forward primer	1	1	1	1	1
25μM Reverse primer	1	1	1	1	1
5U/μL Taq polymerase	0.5	0.5	0.5	0.5	0.5
DNA template	2	2	2	2	2
Sterile water	79.5	79.5	80.5	77.5	77.5
Total volume	**100**	**100**	**100**	**100**	**100**
Annealing temp (^oC)	**54**	**54**	**54**	**68**	**54**

[a] Volumes of each reagent at stated concentrations required for 100 μL reaction. Optimised annealing temperatures are also mentioned.

A PCR reaction lacking the DNA template was also prepared as a negative control. Following PCR, 5μl of the products from each reaction tube were resolved on a standard 1.5% agarose gel. A 100bp standard DNA marker (Promega Ltd) was also loaded on the gel for verification of PCR product sizes.

2.3.7 Purification of DNA

Two different methods for purification of PCR products were investigated. They included the use of SIGMA ALDRICH® GenElute PCR clean up kit (Sigma Aldrich Co.) according to the manufacturer's instructions and the use of a gel extraction kit (QIAGEN).

For the purposes of purifying DNA by the gel extraction method, the DNA samples (25μl) were first analysed on a 0.8% agarose gel. The gel was run at a constant voltage of 60V for 120 minutes. The gel was subsequently placed for 10 minutes in staining solution that contained ethidium bromide in gel running buffer, after which it was placed in destaining solution (sterile distilled water). The gel fragment to be purified was excised from the agarose gel under UV light using a sterile, sharp

scalpel and placed in an eppendorf tube. The weight of the tube before and after addition of the gel slice was noted. The DNA fragment was then purified using the QIAquick® Gel Extraction kit (Qiagen Ltd) according to the manufacturer's instructions.

The purified products were subjected to ethanol precipitation to concentrate the DNA. The purified products were then analysed by agarose gel electrophoresis on a standard 1.5% agarose gel to examine recovery of pure products.

2.3.8 Agarose gel electrophoresis

Agarose gel electrophoresis was used for DNA analysis. For gDNA, a standard 0.8% ($^{w}/_{v}$) agarose gel containing 0.24g agarose (Fischer Scientific Ltd) in 30 mL gel running buffer was prepared. For PCR products, a standard 2% ($^{w}/_{v}$) agarose gel containing 0.6g agarose in 30 mL gel running buffer was prepared. For plasmids, a standard 0.8% ($^{w}/_{v}$) agarose gel containing 0.24g agarose in 30 mL gel running buffer was prepared. The gel was placed in a gel tank containing 300 mL of gel running buffer. DNA samples were prepared by adding gel loading dye at a ratio of 5:1 respectively and loaded onto the gels as carefully as possible. Standard DNA markers (1kb and 100bp represented by M_1 and M_2 respectively) (Promega Ltd), depending on the molecular weight of the expected fragment, were also loaded onto the gel. The gels were run at 100V for approximately 60 minutes. The gels were subsequently stained with ethidium bromide (250 mL of 1 x Gel running buffer contained 50 μL of 5 mg/mL ethidium bromide) for 15 minutes and then de-stained with sterile distilled water for 5 minutes before visualizing under UV radiation on a UV-transilluminator (Ingenius Syngene Bioimaging).

2.3.9 Plasmid isolation

E. coli DH5α cells containing the promoterless pBR322-*lux* plasmids and *E. coli* DH5α cells containing the pSB417 plasmids (all provided by Dr. David Naseby, University of Hertfordshire) were grown by inoculating a single isolated colony of each of the strains into culture flasks containing 5 mL of LB (Luria-Bertani) broth and 100 μg/mL ampicillin under sterile conditions and incubated in a shaking incubator (200rpm) at 37^{o}C for 24 hours. *E. coli* DH5α cells containing pME4510 plasmids were grown by inoculating a single isolated colony of each of the strains into culture flasks containing 5 mL of LB (Luria-Bertani) broth and 10 μg/mL gentamicin under sterile conditions and incubated in a shaking incubator (200rpm) at 37^{o}C for 24 hours. Plasmid DNA was extracted from each of the three strains using the QIAprep® Spin Miniprep kit (Qiagen Ltd) according to the manufacturer's instructions. The concentration and purity of plasmid DNA were determined by measuring the absorbance at 230, 260, 280 and 320nm on the Eppendorf spectrometer. The plasmids were also analyzed by agarose gel electrophoresis on a standard 0.8% agarose gel to examine the purity and conformation of the plasmids.

2.3.10 Restriction

All restriction digest reactions were set up according to the manufacturer's protocols. Restriction endonucleases, restriction buffers and bovine serum albumin (BSA) were purchased from New England Biolabs. The PCR-amplified products were digested with the restriction endonucleases EcoRI and SnaBI simultaneously in a double digestion mix. The plasmid 322-*lux* and pSB417 were digested with EcoRI and BamHI simultaneously in double digests. The reaction mix in each case was prepared according to Table 2.7.

Table 2.7: Restriction digestion components.

	Volumes of each component (μL)[a]		
	PCR products	**322-*lux***	**pSB417**
Sterile distilled water	16.3	32.6	32.6
RE buffer x10	2	4	4
BSA (10μg/μL)	0.2	0.4	0.4
Respective DNA (1μg/μL)	1	2	2
EcoRI endonuclease (10U/μL)	1	1	1
BamHI endonuclease (10U/μL)	-	1	1
SnaBI endonuclease (5U/μL)	2	-	-
Total Volume	**20**	**40**	**40**

[a] Required volumes of each reagent at stated concentrations shown.

Each reaction was incubated at 37^{o}C for 1 hour, after which the reactions were inactivated by incubation at 80^{o}C for 10 minutes. Subsequently, 4μl of each reaction mix was analyzed on an agarose gel. A standard 1.5% agarose gel was prepared for PCR products, whereas a standard 0.8% agarose gel was prepared for plasmids. The agarose gels were run as described in the gel electrophoresis protocol (section 2.3.8). After successful restriction, the digested fragments were subjected to the ligation protocol.

2.3.11 Ligation

All ligation reactions were done according to the manufacturer's protocols (Promega Ltd). The ligation reactions were prepared by mixing together the various reagents for ligation (Table 2.8). The digested PCR products and digested plasmids were mixed in a ratio of 10:1.

Table 2.8: Ligation reaction components

Ligation reagent	Volume of reagent (μL) [a]
Sterile distilled water	-
Ligase reaction buffer x5	4
Plasmid	2.5
PCR product	12.5
Total Volume	19

[a] Required volumes of each reagent at stated concentrations mentioned.

Each reaction was incubated at 65^{o}C for 10 minutes and then cooled to 4^{o}C. T4 DNA ligase (1μL of 1U/μL) was added to each 20μL ligation reaction. The reactions were then incubated at 18^{o}C for 24 hours, after which the reactions were inactivated by incubation at 65^{o}C for 10 minutes. Subsequently 10μl of each reaction mix was analyzed on a standard 0.8% agarose gel (section 2.3.8). After successful ligation, the ligated products were stored at -20^{o}C or prepared for transformation.

2.3.12 Preparation of electro-competent cells

Electro-competent *E. coli* DH5α bacterial cells were prepared as described by Choi, Kumar & Schweizer (2006). Electro-competent *P. aeruginosa* ATCC 9027 bacterial cells were prepared as described by Farinha & Kropinski (1990) and Diver, Bryan & Sokol (1990). However, slight modifications were made. An isolated colony of *P. aeruginosa* cells was inoculated from a working agar plate into 10 mL of LB broth in a

universal bottle and incubated for 24 hours in a shaking incubator at 30^{o}C and 250rpm. The culture (1 mL) was further inoculated into a culture flask containing 100 mL LB broth and incubated for 8 hours in a shaking incubator at 30^{o}C and 250rpm until the O.D. of 0.8 was obtained. The bacterial culture was then treated with alginate lyase (1.28 U/mL) as described by Shen, Han, Lin, & Yu (2006) and incubated at 40^{o}C for 20 minutes. The bacterial culture was then cooled at 4^{o}C for 10 minutes. All further centrifugation and cell-washing steps were done at 4^{o}C. The resultant culture was centrifuged at 3500g for 10 minutes. The resulting supernatant was discarded. The bacterial pellet was re-suspended in 100 mL sterile MilliQ water and then re-centrifuged at 3500g for 10 minutes. The resultant pellet was resuspended in 50 mL of sterile MilliQ water and then re-centrifuged at 3500g for 10 minutes. The pellet was then washed twice in 50 mL of sterile electroporation buffer before re-centrifuging at 3500g for 10 minutes. After the final centrifugation, the pellet was resuspended in 2 mL of sterile electroporation buffer. The suspension was aliquoted into 100 μL aliquots and stored at -70^{o}C until required or used directly for electroporation.

Various electroporation buffers were used for the preparation of electro-competent cells: 300 mM sucrose, 10% glycerol, 1 mM HEPES buffer, Ringers solution, 15% glycerol containing 1 mM MOPS, and 1 mM HEPES pH 7.0 containing 300 mM sucrose and 1 mM magnesium chloride (SMEB).

2.3.13 Electroporation

Electrocompetent cells (40 μl) were thawed on ice before adding 3 μl of plasmid DNA. The cells were mixed gently before transferring them into an ice-cold electroporation cuvette which had a gap of 2 mm. The Bio-Rad Gene Pulser electroporation unit was set at 2.5 kV, 200 Ω resistance and 25 μF capacitance. The cuvette was placed in the unit and an electric pulse was delivered. Immediately after the pulse, 1 mL of SOC media was

added to the cuvette. The cell culture was transferred into a sterile universal tube and incubated at 37^{o}C and 150rpm for 1 hour. An aliquot of the culture (100 μL) of the cells was plated on an LB agar plate containing 10 μg/mL gentamicin. The inoculated agar plate was then incubated in an inverted position at 37^{o}C for 24 hours until colonies appeared. An aliquot of electro-competent cells lacking the addition of plasmid DNA was also pulsed as a negative control. Electroporated *P. aeruginosa* cell cultures were incubated at 30^{o}C instead of 37^{o}C.

2.3.14 Screening of transformants

Each transformed colony was inoculated onto a secondary LB agar plate containing 10 μg/mL gentamicin and also into a culture flask containing 5 mL of LB broth and 10 μg/mL gentamicin and incubated for 16 hours at 37^{o}C. Upon growth, a single isolated colony from the secondary plate was then inoculated into a culture flask containing 5 mL of LB broth and 10 μg/mL gentamicin and incubated for 24 hours at 37^{o}C in a shaking incubator shaking at 250rpm for the purposes of plasmid isolation. All incubations were done at 30^{o}C for *P. aeruginosa* cells. Plasmids were isolated from each of the cultures (section 2.3.9). A small aliquot (2 μL) was analyzed on a standard 0.8% agarose gel. Another aliquot (10 μL) of the plasmid sample was subjected to restriction analysis as described in section 2.3.10. A small sample (5 μL) of the restriction reaction was also analyzed on a standard 0.8% agarose gel to examine the presence of the inserted region. Another plasmid aliquot was subjected to PCR amplification as described in section 2.3.6 to amplify the promoter region if it had been inserted into the plasmid.

2.3.15 Bioluminescence measurement

Each transformed colony was inoculated into 9 mL LB broth containing 10 μg/mL gentamicin and incubated for 24 hours (37°C for *E. coli* and 30°C for *P. aeruginosa*). The cultures (1 mL) were placed into sterile culture tubes and placed into the Celsis Advance Luminometer. The luminometer was programmed to measure relative light units (RLU) of the samples at 30°C. Sterile LB broth (1 mL) was used as a negative control for the measurement of bioluminescence. The lowest limit of detection was pre-set to be 200 RLU.

2.3.16 DNA sequencing

Transformed *E. coli* colonies that were found to contain the plasmids of interest together with *lux* insert and promoter inserts were sent for DNA sequencing to GATC Biotech, to assess the nucleotide sequence of the inserts. Since the *lux* cassette was too long to be sequenced, only the 5'-end of *luxC* and the 3'-end of *luxE* were sequenced. The primers designed to sequence the promoter regions, the *luxC* link at the EcoRI restriction site and the *luxE* link at the BamHI restriction site are listed in Table 2.9:

Table 2.9: Forward and reverse primers for sequencing of insert junctions.

Primer Name [a]	**Primer**
pME-EcoRI F	5'-CTTCATTTTTCCATCTTTGC-3'
LuxC R	5'-TCGGGATAATTAGCAGAGAA-3'
pME-BamHI F	5'-TGTCCATTTTAGCCTCCTTA-3'
LuxE R	5'-TATCTGTTGTTTGTCGGTGA-3'

[a] F denotes forward primer and R denotes reverse primer.

Stock primer suspensions were prepared by adding 100 μL of Tris buffer pH 8. Working solutions of primers were prepared by adding appropriate volumes of tris buffer pH 8, resulting in a DNA concentration of 25 μM. The stocks and the working solutions were stored at -20°C until required.

Working solutions were further diluted to a concentration of 10 pM as required by GATC Biotech.

2.3.17 Long-term storage of all strains

Stock cultures of *P. aeruginosa* ATCC 9027 bioluminescent strains and negative controls were inoculated into vials of bacterial preservation systems containing treated beads in cryo fluid (Fisher Scientific UK Ltd) according to manufacturer's protocols and stored at -70^{o}C for long-term storage. LB agar slopes and plates, with/without 10 μg/mL gentamicin were streaked from the treated beads and incubated at 30^{o}C for 24 hours to create master slopes and plates, respectively. Each strain from the master plate was inoculated onto a working LB agar plate with/without 10 μg/mL gentamicin and incubated for 16 hours at 30^{o}C and used for further applications.

2.3.18 Data analysis

Relative light units and CFU counts were tabulated using Microsoft Excel. Mean values and sample standard deviations were calculated using the built-in functions of Excel. Both RLU readings and CFU counts were converted to Log units. A scatter diagram was plotted for determining the relationship between RLU reading and CFU count. The ratio of Log RLU: Log CFU was also calculated and a column chart was plotted against all serial dilutions. Pearsons' correlations (R-values) were determined, if any, for RLU against CFU using general correlation formulas found in MS Excel.

2.4 Results

2.4.1 Gram staining

P. aeruginosa cells were grown on media to create stocks and working cultures. A characteristic blue-green colour was observed in all cultures containing these species. Upon gram staining, pink rod shaped bacterial cells of size 0.5μm in width and 2μm in length were observed.

2.4.2 Antibiotic susceptibility

P. aeruginosa ATCC9027 cells were found to show resistance against most of the antibiotics tested, as no clear inhibition zones were observed (Table 2.10). However, clear, and well-defined zones were observed in the presence of gentamicin and polymixin B, indicating susceptibility to these antibiotics. Zones of inhibition observed in the presence of streptomycin and nalidixic acid were too small to classify the cells as susceptible.

Table 2.10: Susceptibility of P. aeruginosa *cells to various antibiotics.*

Antibiotic	Concentration (μg/disc)	Zone of inhibition (mm) [a]			Susceptibility
Polymixin B	300	14	15	15	Susceptible
Tetracycline	30	0	0	0	Resistant
Trimethoprim	5	0	0	0	Resistant
Erythromycin	15	0	0	0	Resistant
Nalidixic acid	30	9	9	8	Intermediate
Gentamicin	10	15	16	17	Susceptible
Chloramphenicol	30	0	0	0	Resistant
Streptomycin	10	11	8	9	Intermediate
Kanamycin	5	0	0	0	Resistant
Vancomycin	30	0	0	0	Resistant
Methicillin	5	0	0	0	Resistant
Clindamycin	2	0	0	0	Resistant

[a] Diameters of zones of inhibition indicate whether *P. aeruginosa* was resistant, intermediate or susceptible to stated concentrations of different antibiotics.

2.4.3 Bioinformatics

The gene sequences for each of the genes tat, lysS, lpp, ldcC and spc were retrieved from the genomic DNA sequence of *P. aeruginosa* (Genbank accession number AE004091 version 2) and are presented in Appendix 1. Identification and characterization of the genes within the retrieved sequences was performed using the database MicrobesOnline (Price, Huang, Alm & Arkin, 2005). The start and stop position of each gene, together with the strand on which transcription occurs is detailed in Table 7.1 in Appendix 2. This table also shows the gene transcribed and the translated product. Table 7.2 in Appendix 2 predicts whether the genes lie in the same operon and gives the confidence of the prediction. From these tables, it was shown that the genes for outer membrane lipoprotein (lolB or lpp), lysine decarboxylase (ldcC) and the spc operon were transcribed on the negative strand while the genes for lysyl-tRNA synthetase (lysS) and twin arginine translocase (tat) were transcribed on the positive strand. Table 7.2 in Appendix 2 also indicates the various genes in the spc operon and the tat operon. The statistical prediction value (bOp) indicates whether the gene lies in the same operon as the next gene or in a different operon. In the spc operon, the first gene was rplQ and the last gene was rpsL. Similarly, the gene hisI was predicted to be the first gene in the tat operon and tatC the last. The gene lolB (lpp) was predicted to be located in the same operon as the ipk gene. The genes lysS and ldcC were found not to be part of any operons.

The promoter regions, predicted with BPROM from Softberry Inc., were highlighted in the gene sequences. These regions were compared with the promoter regions for the same genes in *E. coli* (Table 2.11). The nucleotide sequences for the five promoters in the two species *E. coli* and *P. aeruginosa* were found to be dissimilar.

Table 2.11: Promoter sequences for five genes in E. coli *and* P. aeruginosa.

Promoter	Position	*E. coli*	*P. aeruginosa*
ldcC	-10	GGCTATGAT	ATGTATAAA
	-35	TTTTTA	CTGCTG
lpp	-10	ATAAAAAAT	TGTCATGAT
	-35	TTTAAT	TGGGCT
lysS	-10	AAGAAAAAT	GGGCATTGT
	-35	TTTATG	TTTCCT
spc	-10	TGTTATAAT	CGATAAATT
	-35	TTTCTA	TTTCAG
tat	-10	ACGTATAAT	TGGCACACT
	-35	TTCATC	TAGCCA

Specific forward and reverse primers were designed (Table 2.12) to amplify the promoter regions for each of the five genes. All the forward primers were tagged with an EcoRI restriction site at the 5'-end, while all the reverse primers were tagged with a SalI restriction site at the 5'-end.

Table 2.12: Specific primers for amplification of the promoter elements.

Primer Name [a]	Primer	Primer length (bases)[b]	% GC[c]	Tm (^oC)[d]
ldcC F	5'-CTTCAAGAATTCGGGTTACGCGCGTGCCGG-3'	30	60	67
ldcC R	5'-CTGATGTCGACGTCAGTGGGTGGAAGCGACGATGCG-3'	36	55.6	69
lpp F	5'-CTTCAAGAATTCGTATTGACCCCATAGACAGCTTCG-3'	36	44.4	64
lpp R	5'-CTGATGTCGACGTCAGTCTTGTGGATAAGTGACTGGCAG-3'	39	46.2	67
lysS F	5'-CTTCAAGAATTCGGTGTCGGTGATGAGATCGTCG-3'	34	50	66
lysS R	5'-CTGATGTCGACGTCAGCCTATTCCGAGCTGAACGAC-3'	36	50	67
spc F	5'-CTTCAAGAATTCGTAACGCGCGGAACTTCCATC-3'	33	48.5	64
spc R	5'-CTGATGTCGACGTCAAGGTAGATCACCATGGCACGA-3'	36	52.8	68
tat F	5'-CTTCAAGAATTCGGGGTATTCCTGATCCTGCGCCG-3'	35	54.3	68
tat R	5'-CTGATGTCGACGTCACGGCGTTCCAGTGAATCTC-3'	38	50	68

[a] F denotes forward primer and R denotes reverse primer. Underlined sequences show attached restriction sites. The primer length[b] is between 30-40bp, % GC content[c] is around 50% and the melting temperature[d] for each primer is between 60-70^{o}C.

2.4.4 Genomic DNA extraction

Genomic DNA was extracted from *P. aeruginosa* cells. A sharp band of high molecular weight indicated the presence of genomic DNA (gDNA) (Figure 2.3). However, a smear of DNA of low molecular weight indicated the presence of nucleic acid contaminants in the samples (Figure 2.3A). The gDNA samples were then subjected to ethanol precipitation treatment in an attempt to purify the gDNA from the contaminating nucleic acid, and re-analyzed by agarose gel electrophoresis. A sharp DNA band of high molecular weight was observed after the precipitation process (Figure 2.3B), however, the low molecular weight DNA smear of contaminating nucleic acid had disappeared, indicating that the contaminants had been precipitated out.

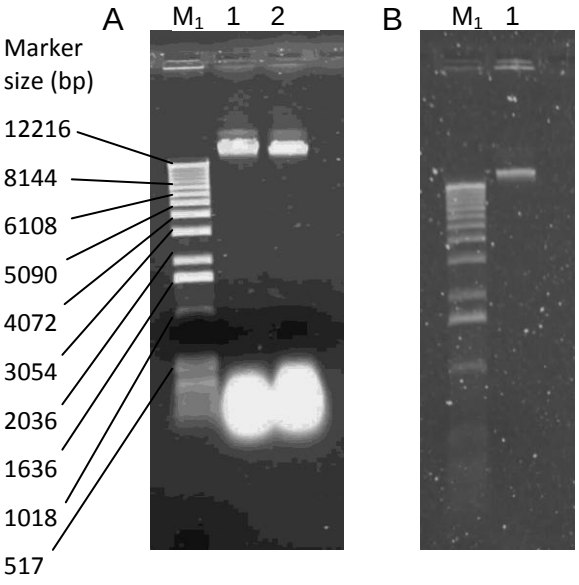


Figure 2.3: Agarose gel electrophoretic analysis of genomic DNA extracted from *P aeruginosa*. A: Lane 1 and 2: genomic DNA samples extracted in duplicate. B: Lane 1: genomic DNA sample after ethanol precipitation.

The purity and concentration of the genomic DNA extracts were estimated by measuring the absorbance at various wavelengths; 230, 260, 280 and 320nm (Table 2.13). Values of at least 1.8 were expected for A ($^{260}/_{280}$) and A ($^{260}/_{230}$). The specified absorbance ratios for samples 1 and 2 were found to be less than 1.8; indicating that the purity of both samples was low. Analysing these ratios further, it was observed that the contaminating factor was proteins since the absorbance at 280nm was high. Hence, the samples were precipitated with the aid of ethanol. Following ethanol precipitation, absorbance ratios of 1.85 for A ($^{260}/_{280}$) and 5 for A ($^{260}/_{230}$) were achieved; indicating that the DNA was pure. However, a lot of DNA was lost during the precipitation process, indicated by the reduction in band intensity and concentration before and after ethanol precipitation.

Table 2.13: Purity and concentration of extracted gDNA samples.

Sample Name	**Before ethanol precipitation**		**After ethanol precipitation**
A ($^{260}/_{280}$)	1.49	1.21	1.85
A ($^{260}/_{230}$)	1.77	1.26	5
A_{230}	0.338	0.941	0.011
A_{260}	0.597	1.19	0.057
A_{280}	0.401	0.985	0.031
A_{320}	0	0.561	0.001
Concentration (μg/mL)	**1195**	**2379**	**114**

Absorbance was measured at 230nm, 260nm, 280nm and 320nm. Absorbance ratios A($^{260}/_{280}$) and A($^{260}/_{230}$) and the concentrations were calculated by the spectrophotometer.

Following gDNA extraction, the purified gDNA was used as a template for downstream polymerase chain reactions.

2.4.5 Polymerase Chain Reaction (PCR)

Expected sequences and sizes for amplicons are shown in Appendix 3.

Several PCR reactions were done for the amplification of the promoter regions and the results of the optimisation of PCR conditions are shown in Appendix 4. Amplification of P_{tat} was done at various annealing temperatures and $MgCl_2$ concentrations (Appendix 4, Figure 7.1). No P_{tat} amplicons were observed in lanes 17-20 (0.25mM $MgCl_2$ and annealing temperatures of 53^{o}C, 56^{o}C, 58^{o}C and 60^{o}C). Two bands for P_{tat} amplicons of approximately 100bp and 200bp were observed in lanes 21 and 23 (0.75mM $MgCl_2$ and annealing temperature of 53^{o}C and 58^{o}C). However, only one band for P_{tat} amplicon of approximately 100bp was observed in lanes 22 and 24 (0.75mM $MgCl_2$ and annealing temperatures of 56^{o}C and 60^{o}C).

Further amplification of tat (Appendix 4, Figure 7.2) revealed bright bands of approximately 175bp for the P_{tat} amplicon in lanes 1-8 (1mM and 1.5mM $MgCl_2$ and annealing temperatures of 53^{o}C, 56^{o}C, 58^{o}C and 60^{o}C).

No P_{lpp} amplicons (Appendix 4, Figure 7.2) were observed in lanes 9-15 (0.25mM and 0.75mM $MgCl_2$). A band for P_{lpp} amplicon of less than 100bp was observed in lanes 16 (0.75mM $MgCl_2$, annealing temperature of 54^{o}C), 17 and 19 (1mM $MgCl_2$, annealing temperatures of 48^{o}C and 52^{o}C, respectively) and lane 23 (1.5mM $MgCl_2$, annealing temperature of 52^{o}C). A bright band for P_{lpp} amplicon of approximately 200bp was observed in lanes 18 and 20 (1mM $MgCl_2$, annealing temperatures of 50^{o}C and 54^{o}C, respectively).

Amplification of P_{ldcC} using the primer pair ldcC F and ldcC R (Appendix 4, Figure 7.3) revealed a bright band of approximately 275bp in lanes 1-6 (0.75mM and 1mM $MgCl_2$ and annealing temperatures of 50^{o}C, 52^{o}C, 54^{o}C and 56^{o}C). A diffuse band of approximately the same size was observed in lanes 7 and 8 (1mM $MgCl_2$ and annealing temperatures of 54^{o}C and 56^{o}C, respectively). Several bands were observed in lanes 9-12

(1.5mM and annealing temperatures of 50^{o}C, 52^{o}C, 54^{o}C and 56^{o}C). This indicated that there was non-specific amplification. The samples in lanes 7, 8 and 9 did not resolve as expected; hence these samples were re-analyzed on a gel (Appendix 4, Figure 7.4). A bright band of approximately 275bp was observed in lanes 1-6 (0.75mM and 1mM $MgCl_2$ and annealing temperatures of 50^{o}C, 52^{o}C and 54^{o}C). Several bands due to non-specific amplification were observed in lane 7 (1.5mM and annealing temperature of 50^{o}C).

Following optimization of the PCR conditions, a confirmatory PCR using the optimized conditions was done for P_{tat}, P_{lpp} and P_{ldcC}. Sharp bands of approximately 175bp, 200bp and 275bp were obtained for P_{tat}, P_{lpp} and P_{ldcC}, respectively (Figure 2.4). The molecular sizes of the bands obtained for P_{lpp} and P_{ldcC} were as expected; however the band for P_{tat} was smaller than the expected size.

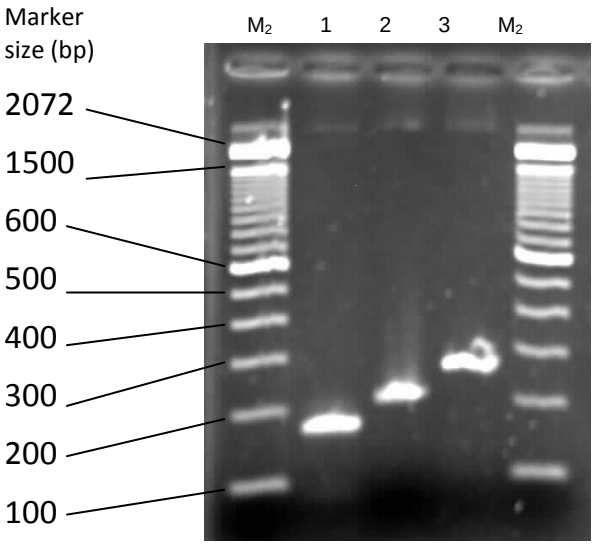


Figure 2.4: Confirmative PCR reactions for P_{tat}, P_{lpp} and P_{ldcC}. The PCR reactions for P_{tat}, P_{lpp} and P_{ldcC} contained 1.5mM $MgCl_2$, 1mM $MgCl_2$ and 1mM $MgCl_2$, respectively, and were done at 54^{o}C.

Several PCR reactions were then done for P_{tat} (Appendix 4, Figure 7.5) at various annealing temperatures and $MgCl_2$ concentrations to optimize the conditions to obtain a band of the correct molecular size. A bright band of under 200bp was observed in all the lanes. Lanes 8-20 also produced

bands of approximately 100bp. This was due to non-specific binding of the primers. A faint band of approximately 450bp was observed in lane 1 and faint bands of less than 300bp were also observed for lanes 11, 12, 16, 17, 19 and 20. No amplicon of the expected size was observed in any of the PCR reactions. The PCR reaction was repeated on an alternative non-gradient PCR machine using the same PCR conditions as before. A band of approximately 200bp was obtained as expected (Figure 2.5).

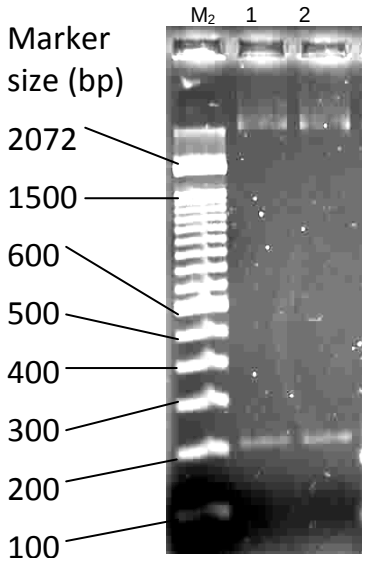


Figure 2.5: Agarose gel electrophoretic analysis of PCR reaction for P_{tat}. The PCR reaction contained 1.5mM $MgCl_2$, and was performed at 54^{o}C.

Amplification of promoter region P_{spc} using the primers pairs spc F and spc R at various annealing temperatures and $MgCl_2$ concentrations (Appendix 4, Figure 7.6) showed P_{spc} amplicons of approximately 125bp in all reactions containing $MgCl_2$ concentrations of 0.75mM, 1mM and 1.5mM and done at annealing temperatures of 50^{o}C, 52^{o}C, 54^{o}C, 56^{o}C and 58^{o}C.

Amplification of promoter region P_{lysS} using the primers pairs lysS F and lysS R at various annealing temperatures and $MgCl_2$ concentrations (Appendix 4, Figure 7.7) resulted in the expected amplicon size of 260bp observed in lanes 2-15 of gel A, all lanes of gel B and C, lanes 1 and 2 of gel D and lanes 5-8 of gel E. However, various other bands indicating non-

specific binding of the primers, were observed in lanes 11-15 of gel A and B, lanes 1-4 of gel C and lanes 1-3 of gel D. The lowest molecular size bands indicated the presence of primer dimers in the reactions.

The PCR conditions for the amplification of each promoter region were summarised (Table 2.14) after optimization of the conditions.

Table 2.14: Optimized PCR reaction conditions for each prototype.

Reagent	**Volume of reagent (μL)**				
	P_{tat}	**P_{lpp}**	**P_{ldcC}**	**P_{lysS}**	**P_{spc}**
10x PCR reaction buffer	10	10	10	10	10
25mM $MgCl_2$ [c]	6	4	4	4	6
25μM Forward primer [a]	1	1	1	1	1
25μM Reverse primer [a]	1	1	1	1	1
10mM dNTP's	2	2	2	2	2
gDNA template	2	2	2	2	2
5U/μL Taq polymerase	0.4	0.4	0.4	0.4	0.4
Sterile distilled water	77.6	79.6	79.6	79.6	77.6
Total	100	100	100	100	100
Annealing temperature [b]	**54°C**	**54°C**	**54°C**	**62°C**	**58°C**

[a] Forward and reverse primers specific to each prototype were used. [b] Optimised annealing temperature and [c]optimised magnesium chloride volume.

2.4.6 Purification of PCR products

PCR reaction products for all five promoter amplicons were then purified using the QIAGEN PCR purification kit and analyzed on a gel (Figure 2.6), once it was ensured that the DNA band was eluted in the correct step during the purification process. Bands of approximately 275bp, 175bp (Figure 2.6A) and 125bp (Figure 2.6B) for purified products P_{lysS}, P_{tat} and P_{spc}, respectively, were observed. Bands of 225bp and 275bp in lanes 1-2 (Figure 2.6C) for purified P_{lpp} and P_{ldcC}, respectively, were observed.

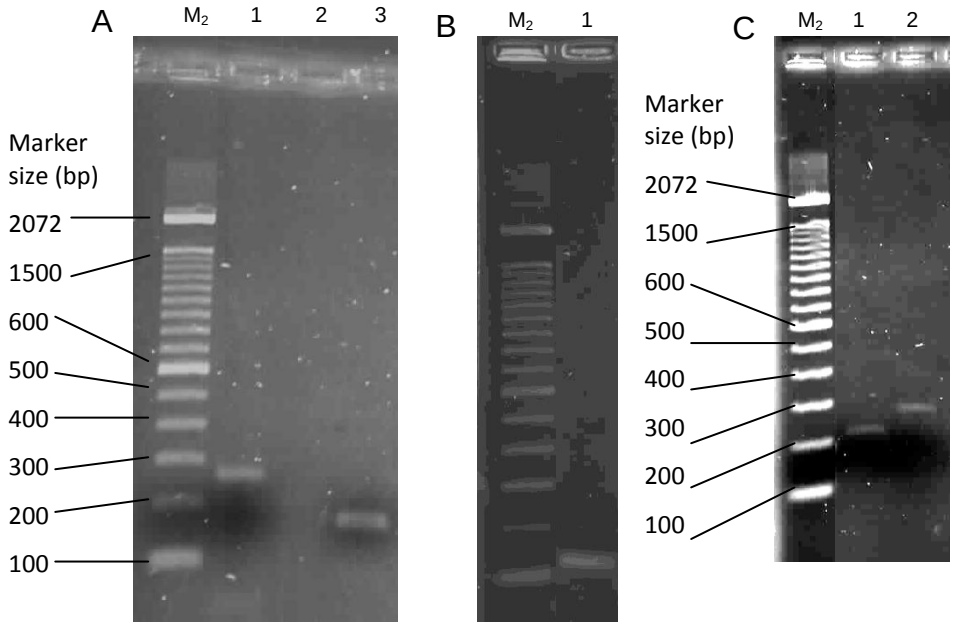


Figure 2.6: Purification of P_{lysS}, P_{tat}, P_{spc}, P_{lpp} and P_{ldcC} amplicons. A: purified P_{lysS} and P_{tat} PCR products. B: purified P_{spc} PCR products. C: purified P_{lpp} and P_{ldcC} in lanes 1-2 respectively.

The purity and concentration of all five purified amplicons were estimated by measuring the absorbance at various wavelengths (Table 2.15). Absorbance ratios greater than 1.7 were obtained.

Table 2.15: Purity and concentration of each prototype after PCR purification.

Sample Name	**P_{lysS}**	**P_{tat}**	**P_{lpp}**	**P_{ldcC}**	**P_{spc}**
A ($^{260}/_{280}$)	1.75	1.75	1.70	1.75	1.68
A ($^{260}/_{230}$)	1.95	1.88	1.74	1.89	1.77
A_{230}	0.706	0.374	0.615	0.765	0.389
A_{260}	1.374	0.704	1.073	1.444	0.687
A_{280}	0.786	0.403	0.632	0.823	0.408
A_{320}	0.071	0.043	0.105	0.081	0.067
Concentration (μg/mL)	**69**	**35**	**54**	**72**	**34**

Absorbance was measured at 230nm, 260nm, 280nm and 320nm. The absorbance ratios A($^{260}/_{280}$) and A($^{260}/_{230}$) and the concentrations were calculated by the spectrophotometer.

2.4.7 Plasmid isolation

Each plasmid, pBR322, pSB417, pME4510 and promoterless pBR322-*lux*, was isolated from its respective *E. coli* strain and analyzed by agarose gel electrophoresis. Bands of different molecular weights were obtained. However, it was difficult to determine the exact size of the plasmid without restriction. The different bands of the plasmid corresponded to the various conformations of the plasmid. Plasmid bands of approximately 3.5kbp were observed for plasmid pBR322 (Figure 2.7A) isolated using the QIAGEN (lanes 1 and 2) and the Sigma kits (lanes 3 and 4). Three plasmid bands of approximately 8kbp, 10kbp and a higher molecular size were observed for plasmid pSB417 isolated using the Sigma kit (Figure 2.7A, lanes 5 & 6). Three plasmid bands of approximately 10kbp and higher molecular sizes were observed for plasmid pBR322-*lux* isolated using the Sigma kit (Figure 2.7B). A band of approximately 3.5kbp was observed for plasmid pME4510 (Figure 2.7C).

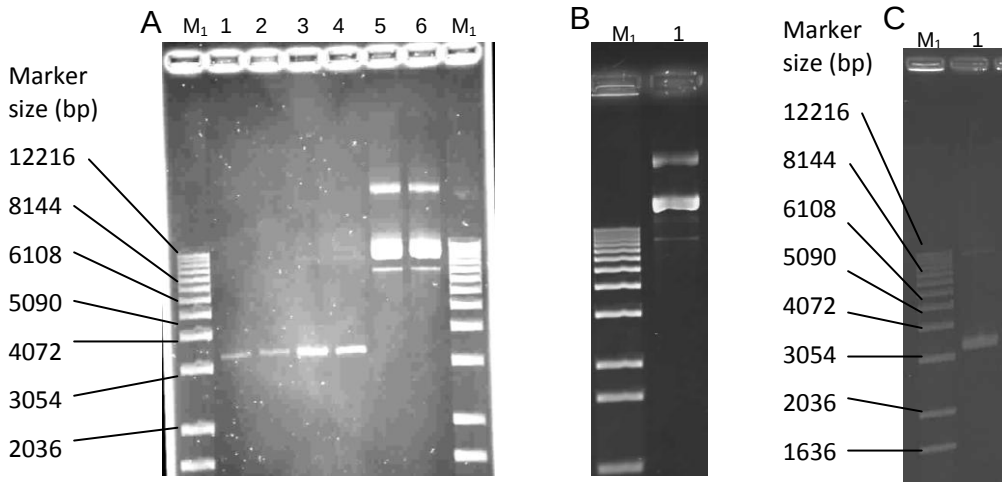


Figure 2.7: Agarose gel electrophoretic analysis for various plasmid isolations. A: Plasmids pBR322 isolated using the Qiagen and Sigma kits were represented in lanes 1-2 and 3-4 respectively. Plasmids pSB417 isolated using the Sigma kit was represented in lanes 5 and 6. B: The promoterless plasmid pBR322-*lux* isolated using the Sigma kit was represented in lane 1. C: The plasmid pME4510 isolated was represented in lane 1.

The purity and concentration of all the isolated plasmids were estimated by measuring the absorbance at various wavelengths (Table 2.16). Absorbance ratios greater than 1.7 for A($^{260}/_{280}$) were obtained. Very low absorbances at 320nm indicated low amounts of salt impurities and therefore high purity of samples. Suitable concentrations of plasmids were obtained for use in further restriction digestions.

Table 2.16: Purity and concentration of isolated plasmids.

Sample Name	**pBR322**	**pSB417**			**pBR322-*lux***			
A ($^{260}/_{280}$)	1.8	1.76	1.70	1.78	1.79	1.80	1.76	1.75
A ($^{260}/_{230}$)	2.16	2.28	2.30	2.54	1.74	1.77	1.84	1.69
A_{230}	0.502	1.060	1.079	0.726	1.047	0.819	1.294	1.371
A_{260}	1.083	2.418	2.487	1.846	1.817	1.451	2.382	2.317
A_{280}	0.602	1.374	1.462	1.035	1.016	0.805	1.353	1.325
A_{320}	0.085	0.043	0.040	0.033	0.005	0.002	0.006	0.013
Concentration (μg/mL)	**54**	**121**	**124**	**92**	**91**	**73**	**119**	**116**

Absorbance was measured at 230nm, 260nm, 280nm and 320nm. The absorbance ratios A ($^{260}/_{280}$) and A($^{260}/_{230}$) and the concentrations were calculated by the spectrophotometer.

2.4.8 Restriction of plasmids pBR322, pSB417, 322-*lux* and pME4510

Double digestion of plasmid pBR322 with EcoRI and PstI (Appendix 4, Figure 7.8) for one hour produced two bands of approximately 3.5kbp and 750bp (lane 2). Double digestion of plasmid pSB417 with SalI and PstI for one hour produced only one band of approximately 8.5kbp (lane 4) instead of two bands.

Absence of the appearance of two bands from plasmid pSB417 restriction with SalI and PstI led to further investigations (Appendix 4, Figure 7.9). Single restriction analysis of plasmid pSB417 with SalI (lane 1) and PstI (lane 2) produced single bands of 5.8kbp instead of the expected two bands. However double restriction analysis of pBR322-*lux* with the same

enzymes SalI and PstI (lane 3) revealed two bands of approximately 6.5kbp and 2.9kbp. Double restriction analysis of pSB417 with EcoRI and BamHI together (lane 5) also produced two bands of approximately 5.8kbp and 2.7kbp. Double restriction analysis of pBR322 with EcoRI and BamHI together (lane 6) produced two bands of approximately 5.8kbp and 3.9kbp.

The absence of the appearance of two bands from plasmid pSB417 restriction with SalI and PstI was confirmed when single digests (Appendix 4, Figure 7.10A) of the plasmid with SalI (lane 2) and PstI (lane 3) revealed single bands of 8.5kbp in each reaction. Further analysis of the restriction of the SalI-restricted fragment with PstI also revealed a single band of 8.5kbp. Restriction analysis of plasmid pSB417 with SalI and XbaI (Appendix 4, Figure 7.10B) revealed that the digestion was incomplete as shown by the presence of undigested plasmid band (8.5kbp). However more importantly, two bands of approximately 3.8kbp and 4.6kbp were also produced.

Double restriction digest of the promoterless plasmid pBR322-*lux* with EcoRI and BamHI (Appendix 4, Figure 7.11A) produced two bands of approximately 3.9kbp and 5.8kbp (lanes 2 and 3). A single restriction digest of the plasmid with SnaBI (Appendix 4, Figure 7.11B) also produced a fragment of approximately 10kbp.

This indicated that the reverse primers for the promoter regions had to be re-designed to incorporate the SnaBI restriction site at the 5'-end instead of the SalI restriction site to allow ligation of the promoter amplicons into the promoterless pBR322-*lux* plasmid. Hence the reverse primers were re-designed while the original forward primers containing the EcoRI restriction site were used. The PCR reactions for all the 5 promoters were re-optimized.

Restriction of the promoterless pBR322-*lux* plasmid with both EcoRI and SnaBI was done successfully (Appendix 4, Figure 7.12). Single restriction digests of the plasmid with SnaBI (lane 2) and EcoRI (lane 4) produced

single fragments of ~10kbp in each reaction (Appendix 4, Figure 7.12A). Single consecutive restriction digests of plasmid with SnaBI first (lane 2 of gel B) and then EcoRI (lane 3 of gel B) also produced a single fragment of ~10kbp. Finally, a double restriction digest of the plasmid with SnaBI and EcoRI also produced a single fragment of ~10kbp (gel C), as expected.

Subsequently, plasmids pSB417 and 322-*lux* were digested with EcoRI and BamHI in double digests (Figure 2.8). Two bands of 5.8kb and 2.5kb were observed in lane 2 following digestion of plasmid pSB417. Similarly, two bands of approximately 5.8kb and 3.8kb were observed in lane 4. The 5.8kbp fragment corresponded to the excised *lux* cassette.

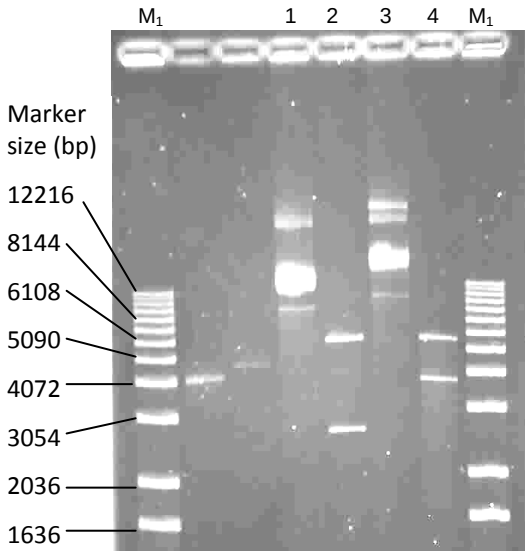


Figure 2.8: Restriction of Plasmids pSB417 and 322-*lux*. Plasmids pSB417 and 322-*lux* digested in double digests with EcoRI and BamHI are represented in lanes 2 and 4 respectively with the isolated plasmids pSB417 and 322-*lux* represented in lanes 1 and 3 respectively.

The plasmid pME4510 was digested with EcoRI and BamHI in a double digest for 1 hour. A band of approximately 4kb for digested plasmid was observed in lane 2 (Figure 2.9). Plasmid pSB417 harbouring the *lux* cassette was also digested by the same procedure as plasmid pME4510. Two bands of approximately 5.8kb and 2.5kb, corresponding to the excised *lux* cassette and remaining plasmid sequence, respectively, were observed in lane 4 following digestion of plasmid pSB417.

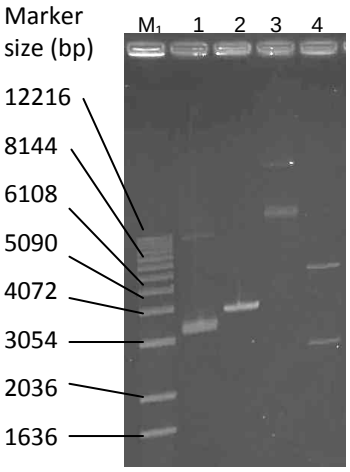


Figure 2.9: Restriction of plasmids pME4510 and pSB417. Undigested and digested plasmid pME4510 are represented in lanes 1 and 2 respectively. Undigested and digested plasmid pSB417 are represented in lanes 3 and 4 respectively. The plasmid was digested by EcoRI and BamHI in a double digest.

2.4.9 Ligation of *lux* cassette into pME4510.

Ligation of the excised *lux* cassette from plasmid pSB417 into the plasmid vector pME4510 (Figure 2.10) was done. Digested pME4510 and pSB417 produced fragments of 3.8kbp (lane 1) and 5.8kbp and 2.7kbp (lane 2) respectively. Ligation of the *lux* cassette from plasmid pSB417 into the plasmid vector was observed by the presence of several bands in lane 3 indicating re-circularization of the recombinant plasmids (Figure 2.10). The presence of a 3.8kbp fragment in lane 3 indicated that excess plasmid vector was also present in the ligation reaction.

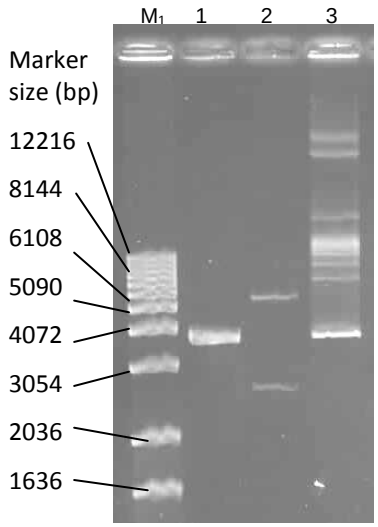


Figure 2.10: Ligation of excised *lux* cassette into plasmid vector pME4510. Restricted plasmids pME4510 and pSB417 are represented in lanes 1 and 2 respectively. Ligation of *lux* cassette from plasmid pSB417 into the plasmid vector is represented in lane 3.

2.4.10 Transformation and screening of pME-*lux*

Transformation of recombinant plasmid pME-*lux* into electro-competent *E. coli* DH5α cells with successful ligation reactions produced over 200 colonies of transformants; of which, 24 colonies were selected at random for screening. Following plasmid isolation from all 24 colonies and digestion to assess the presence of the *lux* cassette, only seven out of 24 recombinant plasmids harboured the *lux* cassette (Lanes 2, 4, 5, 15, 16, 19 and 21) (Figure 2.11). The expected DNA sequence of pME-*lux* is shown in Appendix 5.

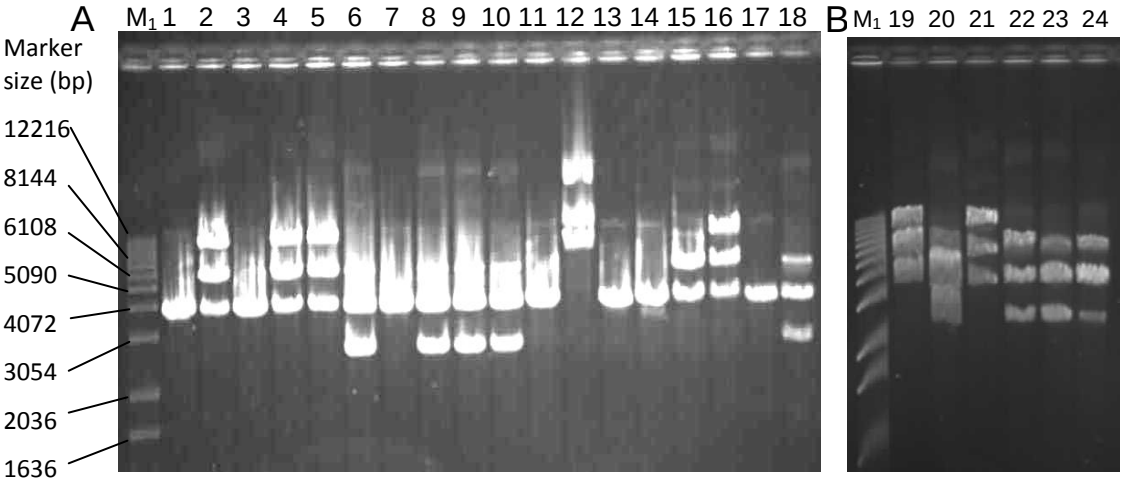


Figure 2.11: Restriction of pME*lux* recombinant plasmids isolated from transformed *E. coli* DH5α cells. The plasmids were digested by EcoRI and BamHI in a double digest.

2.4.11 Restriction of pME-*lux* plasmids

Following successful transformation of the promoterless pME-*lux* plasmids, the next stage was to insert promoters upstream of the *lux* cassette. Restriction analysis was done to assess the ability of restriction endonucleases EcoRI and SnaBI to digest the plasmids (Figure 2.12) because the promoter amplicons were flanked by EcoRI and SnaBI restriction sites. Plasmids from seven isolates that harboured the *lux* cassette in plasmid pME-*lux* were also digested in a double digest with EcoRI and SnaBI to assess the presence of intact restriction sites (Figure 2.12). Plasmids in lanes 1, 2, 3 and 7 produced single fragments of 10kbp and therefore harboured intact EcoRI and SnaBI restriction sites. Lane 5 was replicated in lane 6 in error; hence these two samples are based on the same restriction reaction. Plasmids in lanes 4 and 8 remained undigested, indicating that their restriction sites were non-functional. Plasmids in lanes 5, 6 and 8 produced fragments of incorrect molecular weights.

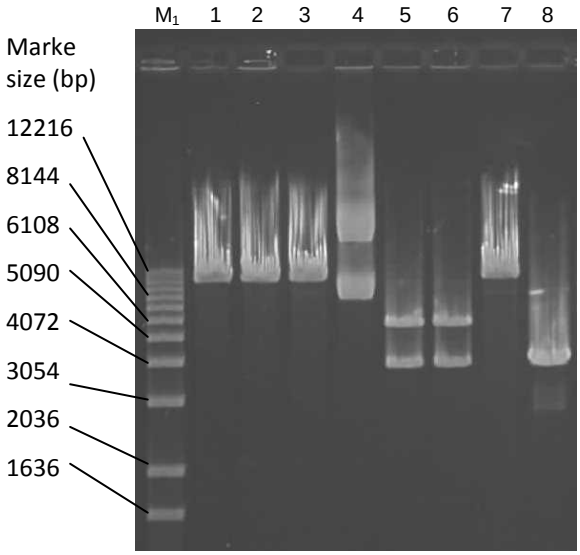


Figure 2.12: Restriction of promoterless plasmid pME-*lux*. Double restriction digests of pME-*lux* with EcoRI and SnaBI.

Plasmid pME-*lux* in lane 1 of Figure 2.12 was subsequently used for all further manipulations. The purity and concentration of the isolated plasmid pME-*lux* was estimated by measuring the absorbance at various wavelengths (Table 2.17). Absorbance ratios greater than 1.7 were obtained.

Table 2.17: Purity and concentration of isolated pME-lux plasmid.

Sample Name	**pME-*lux***
A ($^{260}/_{280}$)	1.79
A ($^{260}/_{230}$)	2.43
A_{230}	1.016
A_{260}	2.470
A_{280}	1.377
A_{320}	0.019
Concentration (μg/mL)	**123**

Absorbance (A) was measured at 230nm, 260nm, 280nm and 320nm. The absorbance ratios A ($^{260}/_{280}$) and A($^{260}/_{230}$) and the concentrations were calculated by the spectrophotometer.

2.4.12 PCR with new primers

New reverse primers were designed in order to replace the SalI restriction site with the SnaBI restriction site (Table 2.18).

Table 2.18: New reverse primers tagged with SnaBI restriction site.

Primer Name [a]	Primer [b]	Primer length (bases) [c]	% GC [d]	Tm (°C) [e]
ldcC F	5'-CTTCAAGAATTCGGGTTACGCGCGTGCCGG-3'	30	60	67
ldcC R	5'-CTGATTACGTAGTCAAGAGAATGCTGAAGCCGTCCTG-3'	37	48.7	67
lpp F	5'-CTTCAAGAATTCGTATTGACCCCATAGACAGCTTCG-3'	36	44.4	64
lpp R	5'-GTGATTACGTAGTCAGCGCAAGGGATTTGTTCATA-3'	35	42.9	63
lysS F	5'-CTTCAAGAATTCGCTGTCTCTGGGAGCTACTCG-3'	33	51.5	66
lysS R	5'-GTGATTACGTAGTCAGATAGCGGCCGATTGATTC-3'	34	47.1	64
spc F	5'-CTTCAAGAATTCGTAACGCGCGGAACTTCCATC-3'	33	48.5	64
spc R	5'-CTCATTACGTAGTCAAGGTAGATCACCATGGCACGA-3'	36	47.2	66
tat F	5'-CTTCAAGAATTCGGGGTATTCCTGATCCTGCGCCG-3'	35	54.3	68
tat R	5'-GTGATTACGTAGTCAGCAACAGTCCATGGAAAAGC-3'	35	45.7	64

[a] F denotes forward primer and R denotes reverse primer. [b] Underlined sequences show attached restriction sites. The [c] primer length is between 30-40bp, % GC content[d] is between 40-60% and the [e] melting temperature for each primer is between 60-70°C.

Based on the promoter region being amplified by the new set of primer pairs, the sizes of the amplicons and the sizes of each amplicon after restriction with EcoRI and SnaBI were calculated (Table 2.19). The nucleotide sequences for the amplicons for each promoter region are represented in Appendix 1.

Table 2.19: Sizes of PCR amplicons.

	PCR products using new primers[a]	
	Size	Size after restriction
lysS	125	110
tat	206	191
lpp	161	146
ldcC	225	210
spc	134	119

[a]Sizes of PCR amplicons before and after digestion with EcoRI and SnaBI.

Several PCR reactions were then done for the amplification of each promoter region P_{tat}, P_{lpp}, P_{ldcC}, P_{spc} and P_{lysS}, using the primers pairs tat F and tat R, lpp F and lpp R, ldcC F and ldcC R, spc F and spc R, lysS F and lysS R respectively. The reactions were done at various annealing temperatures ranging from 52^{o}C to 68^{o}C and $MgCl_2$ concentrations ranging from 0.75mM to 1.5mM.

A confirmatory PCR using the optimized conditions was performed for the amplification of all five promoter regions (Figure 2.13). Bands of approximately 225bp (lane 1), 160bp (lane 2), 200bp (lane 3), 125bp (lane 4) and 135bp (lane 5) were obtained for P_{ldcC}, P_{lpp}, P_{tat}, P_{lysS} and P_{spc}, respectively.

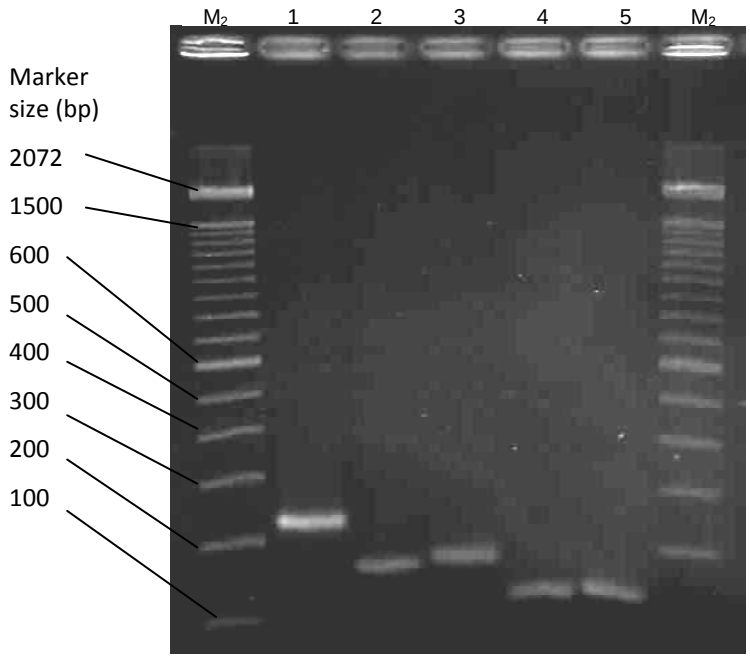


Figure 2.13: PCR reactions for P_{ldcC}, P_{lpp}, P_{tat}, P_{lyss} and P_{spc} as represented in lanes 1, 2, 3, 4 and 5 respectively.

The purity and concentration of all five purified PCR products were estimated by measuring the absorbance at various wavelengths (Table 2.20). Absorbance ratios greater than 1.7 for A($^{260}/_{280}$) and A ($^{260}/_{280}$) were obtained, as expected. Furthermore, the absorbance ratios for A($^{260}/_{230}$) were greater than 2. Very low absorbances at 320nm indicated low

amounts of particles in the solution and therefore high purity of samples. High concentrations of PCR products were obtained and this indicated that the purified PCR products could be used for further restriction digestions.

Table 2.20: Purity and concentration of purified PCR products.

Sample Name	P_{ldcC}	P_{lpp}	P_{tat}	P_{lysS}	P_{spc}
A ($^{260}/_{280}$)	1.8	1.79	1.78	1.77	1.77
A ($^{260}/_{230}$)	2.11	2.18	2.08	2.14	2.21
A_{230}	0.712	0.476	0.766	0.443	0.599
A_{260}	1.500	1.037	1.596	0.949	1.322
A_{280}	0.834	0.581	0.897	0.537	0.749
A_{320}	0.028	0.026	0.047	0.025	0.026
Concentration (μg/mL)	**75**	**52**	**80**	**47**	**66**

Absorbance (A) was measured at 230nm, 260nm, 280nm and 320nm. The absorbance ratios A ($^{260}/_{280}$) and A($^{260}/_{230}$) and the concentrations were calculated by the spectrophotometer.

The optimised PCR conditions for each prototype are summarised in Table 2.21.

Table 2.21: Optimized PCR reaction conditions for amplification of all promoter regions.

Reagent	Volume of reagent (μL)				
	P_{tat}	P_{lpp}	P_{ldcC}	P_{lysS}	P_{spc}
10x PCR reaction buffer	10	10	10	10	10
25mM $MgCl_2$	6	4	3	6	6
25μM Forward primer[a]	1	1	1	1	1
25μM Reverse primer[a]	1	1	1	1	1
10mM dNTP's	2	2	2	2	2
gDNA template	2	2	2	2	2
5U/μL Taq polymerase	0.4	0.4	0.4	0.4	0.4
Sterile distilled water	77.6	79.6	80.6	77.6	77.6
Total	100	100	100	100	100
Annealing temperature	**66°C**	**54°C**	**64°C**	**68°C**	**58°C**

[a]Forward and reverse primers specific to each promoter were used.

2.4.13 Restriction of PCR products

The purified PCR products for all five promoter regions were subjected to restriction in a double digest by the restriction endonucleases EcoRI and SnaBI. The digested fragments were then analyzed by agarose gel electrophoresis (Figure 2.14). Restriction of amplicons P_{lysS} (lane 2), P_{ldcC} (lane 4) and P_{tat} (lane 6) with EcoRI and SnaBI produced fragments of approximately 110bp, 210bp and 200bp, respectively (Figure 2.14A). Restriction of amplicons P_{lpp} (lane 2) and P_{spc} (lane 4) (Figure 2.14B) produced fragments of approximately 200bp. There were no observable differences in the mobilities of the undigested and digested fragments on the gel, suggesting that such small differences in low-molecular weight fragments were not resolved well on the gel.

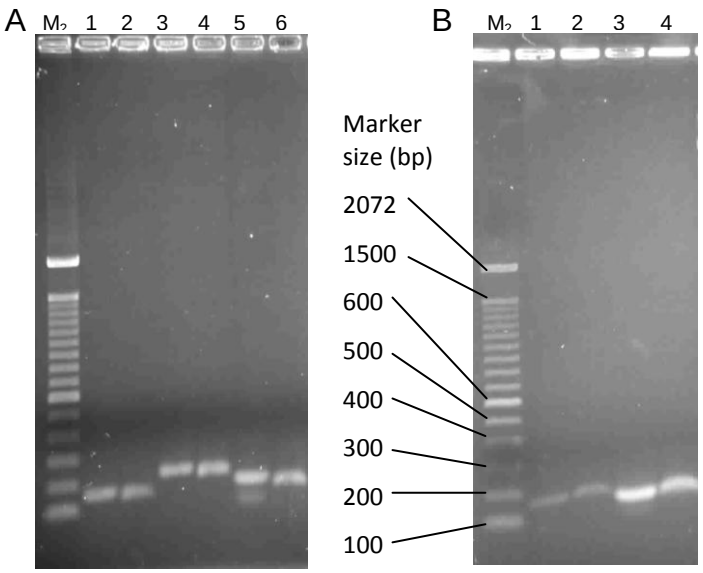


Figure 2.14: Restriction of PCR products. PCR products were digested in a double digest by EcoRI and SnaBI. Figure A: Undigested and digested P_{lysS} is represented in lanes 1 and 2 respectively. Undigested and digested P_{ldcC} is represented in lanes 3 and 4 respectively. Undigested and digested P_{tat} is represented in lanes 5 and 6 respectively. Figure B: Undigested and digested P_{lpp} is represented in lanes 1 and 2 respectively. Undigested and digested P_{spc} is represented in lanes 3 and 4 respectively.

2.4.14 Ligation of PCR products into promoterless pME-*lux* plasmid

Aliquots of ligation reaction products of each digested promoter fragment P_{spc}, P_{ldcC}, P_{lpp}, P_{lysS} and P_{tat} with the restricted promoterless plasmid pME-*lux* (lane 1) were analyzed by agarose gel electrophoresis (Figure 2.15). Bands of ~10kbp corresponding to spc-pME-*lux*, ldcC-pME-*lux*, lpp-pME-*lux*, lysS-pME-*lux* and tat-pME-*lux* in various gels were obtained in all cases. However, several other bands of low molecular sizes were also obtained during the ligation reactions.

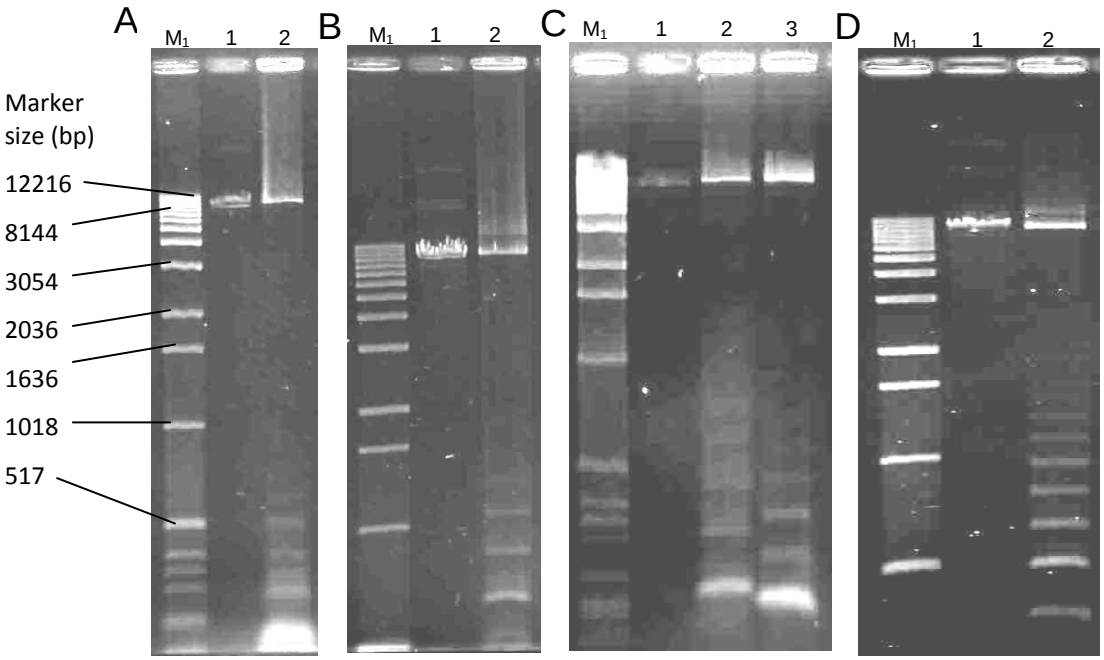


Figure 2.15: Ligation of each of the five restricted amplicons and plasmid pME-*lux*. Promoterless plasmid pME-*lux* digested with EcoRI and SnaBI is shown in lane 1 of all gels. (A) Ligation reaction of P_{spc} with digested promoterless plasmid is represented in lane 2. (B) Ligation reaction of P_{ldcC} with digested plasmid is shown in lane 2. (C) Ligation reaction of P_{lpp} and P_{lysS} with digested plasmid is shown in lanes 2 and 3 respectively. (D) Ligation reaction of P_{tat} with digested plasmid is shown in lane 2.

2.4.15 Transformation and screening of recombinant plasmids from pME-*lux*

Transformation of recombinant plasmids containing the promoter amplicons into electro-competent *E. coli* DH5α cells was successful and the numbers of colonies produced in each case are detailed in Table 2.22.

Table 2.22: E. coli *DH5α bacterial colonies post electroporation.*

Prototype	Number of colonies
No plasmid	0
pME4510	200
pME*lux*	24
ldcC-pME-*lux*	13
lysS-pME-*lux*	11
tat-pME-*lux*	163
spc-pME-*lux*	9
lpp-pME-*lux*	39

Each electroporation mixture contained 3μL ligation mix and 40μL electrocompetent cells.

Successful transformants were selected, based on their ability to grow in the presence of gentamicin. All colonies were sub-cultured onto secondary agar plates as well as into LB broth. Plasmids were isolated from all colonies and digested with EcoRI and SnaBI to assess the presence of the amplicons. Following plasmid isolations and restriction analysis to assess the presence of promoter amplicons, a confirmatory gel was run to confirm the presence of the promoter regions (Figure 2.16). Three out of 12 colonies were shown to harbour P_{spc}, as represented in lane 2-4. Two out of 10 colonies were found to harbour P_{ldcC}, as represented in lane 5-6. Three out of 8 colonies were found to harbour P_{lysS}, as represented in lanes 7-9. Two out of 10 colonies were found to harbour P_{lpp}, as represented in lanes 10-11. Two out of 6 colonies were found to harbour P_{tat}, as represented in lanes 12-13. Lane 1 of the gel represents restriction of the promoterless pME-*lux* as a negative control.

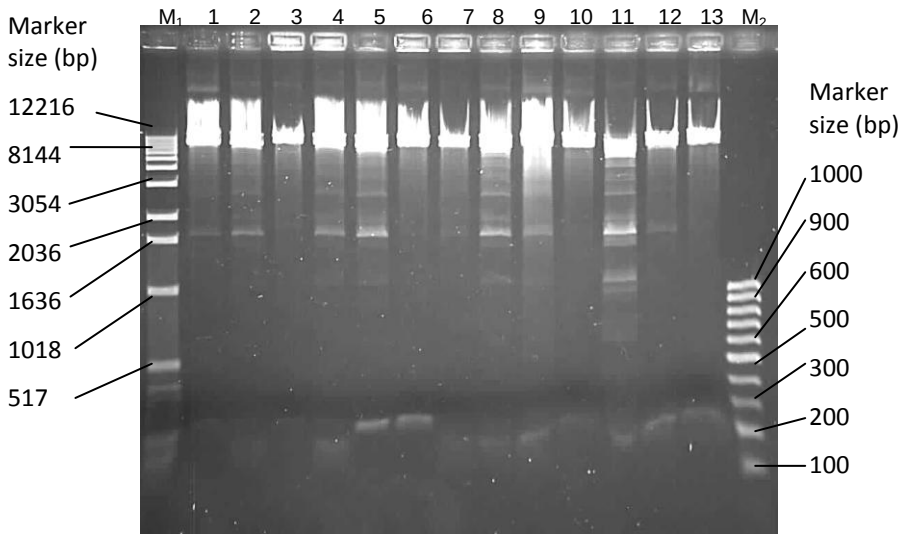


Figure 2.16: Restriction of recombinant plasmids isolated from transformed *E. coli* DH5α cells. Lane 1 represents promoterless pME-*lux*. Lanes 2-4 represent restriction of spc-pME*lux* recombinant plasmids. Lanes 5-6 represent restriction of ldcC-pME-*lux*. Lanes 7-9 represent restriction of lysS-pME-*lux* recombinant plasmids. Lanes 10-11 represent restriction of lpp-pME-*lux* recombinant plasmids. Lanes 12-13 represent restriction of tat-pME-*lux* recombinant plasmids. All plasmids were digested in a double digest with EcoRI and SnaBI.

2.4.16 DNA sequencing of *E. coli* recombinant plasmids

All thirteen recombinant plasmids isolated from *E. coli* transformant strains were sent for DNA sequencing to analyse the nucleotide sequence for inserted promoter and *lux* cassette regions. All plasmids were found to harbour the defined promoter inserts between EcoRI and SnaBI restriction sites. Similarly, all plasmids were found to harbour the *lux* cassette between EcoRI and BamHI restriction sites. DNA sequences for each of the thirteen plasmid isolates are represented in Appendix 6.

2.4.17 Transformation into *P. aeruginosa* ATCC 9027

Transformation of recombinant plasmids containing the promoter amplicons and the *lux* cassette into electro-competent *P. aeruginosa* ATCC 9027 cells was attempted using a variety of electroporation buffers. No transformant colonies were obtained on selective media when 300mM sucrose, 10% glycerol or 25mM HEPES buffer containing 300mM glucose and 5mM calcium chloride was used as the electroporation buffer, even after several attempts. Transformation was also unsuccessful when the amount of plasmid was varied, recovery time post-electroporation was varied and when capacitance and resistance parameters on the electroporation equipment were varied.

Following numerous unsuccessful attempts to transform *P. aeruginosa*, a further range of alternative electroporation buffers were used. These included 15% glycerol containing 1mM MOPS, Ringers solution, and 1mM HEPES pH 7.0, containing 300mM sucrose and 1mM magnesium chloride. Ringers solution was most effective as an electroporation buffer, followed by 1mM HEPES pH 7.0, containing 300mM sucrose and 1mM magnesium chloride and lastly 15% glycerol containing 1mM MOPS. The numbers of transformant colonies obtained after screening on selective media are detailed in Table 2.23.

Table 2.23: P. aeruginosa *ATCC 9027 bacterial colonies post electroporation.*

Prototype	**Number of colonies with various electroporation buffers**		
	Ringers solution	Glycerol-MOPS	HEPES-sucrose-$MgCl_2$
No plasmid	0	0	0
pME4510	1	3	18
pME*lux*	7	0	0
ldcC-pME-*lux*	8	1	2
lysS-pME-*lux*	8	12	1
tat-pME-*lux*	3	0	11
spc-pME-*lux*	1	2	
lpp-pME-*lux*	7	0	0
Total	35	18	32

Each electroporation mixture contained 3μL ligation mix and 40μL electrocompetent cells.

All colonies were sub-cultured onto secondary agar plates as well as into LB broth. Each of the transformant colonies was also assessed for bioluminescence ability by measuring relative light units (Table 2.24) and comparing to the negative controls pME4510 and pME*lux*.

Table 2.24: Relative Light Units (RLU's) from P. aeruginosa *transformants***.**

Plasmid isolate from *E. coli*	***P. aeruginosa* transformant**	**Mean RLU**		**Plasmid isolate from *E.coli***	***P. aeruginosa* transformant**	**Mean RLU**
pME4510	H14	90		**pME*lux***	R3	79
	H15	90			R9	90
	H21	85			R13	88
	H23	91			R15	77
	H31	111			R23	100
	H41	102			R28	87
	H42	110			R29	82
	G2	91		**spc3**	R1	81
	G3	89		**spc5**	G1a	280579
	G4	88			G1b	96
	R2	92		**ldcC8**	H1a	645355
lpp4	R1	90			H1b	110
lpp4	R1	86		**ldcC4b**	G1	86
	R3	553431			R1	84
	R4	3036426			R9	85
	R6	191			R15	91
	R11	85			R17	88
lys6	R8	101			R20	87
	R10	2252009			R21	84
	R14	2519292			R22	92
	R25	5307196			R23	92
	R26	271		**lpp8**	R3	96
	R27	816220		**tat9**	H1	80
	R28	114			H2	87
	R29	2090			R11	89
	G1	743591			R12	1042124
	G4	1651257			H5	3798007
	G8	2487095			H8	916
	G10	2142399			H9	1108108
	G14	64984			H11	292688
	G16	90			H12	703611
	G17	624			H13	318533
	G22	100			H14	2103569
	G25	2790726		**tat12**	R1	149
	G27	674549			H8	6277
	G28	4120437			H10	1218121
	G29	178553		**lys3**	H1	97

The letters G, R and H next to the *P. aeruginosa* transformant number represents glycerol containing MOPS, ringers solution and sucrose containing HEPES respectively; the type of electroporation media used during preparation of electro-competent cells.

Plasmids were isolated from transformants that produced bioluminescence greater than a threshold value of 200 RLU's from Table 2.24 (Figure 2.17). Transformants harbouring plasmids pME4510 and pME-*lux* as negative controls were not expected to produce bioluminescence and therefore transformants that failed to produce bioluminescence were also subjected to plasmid isolation.

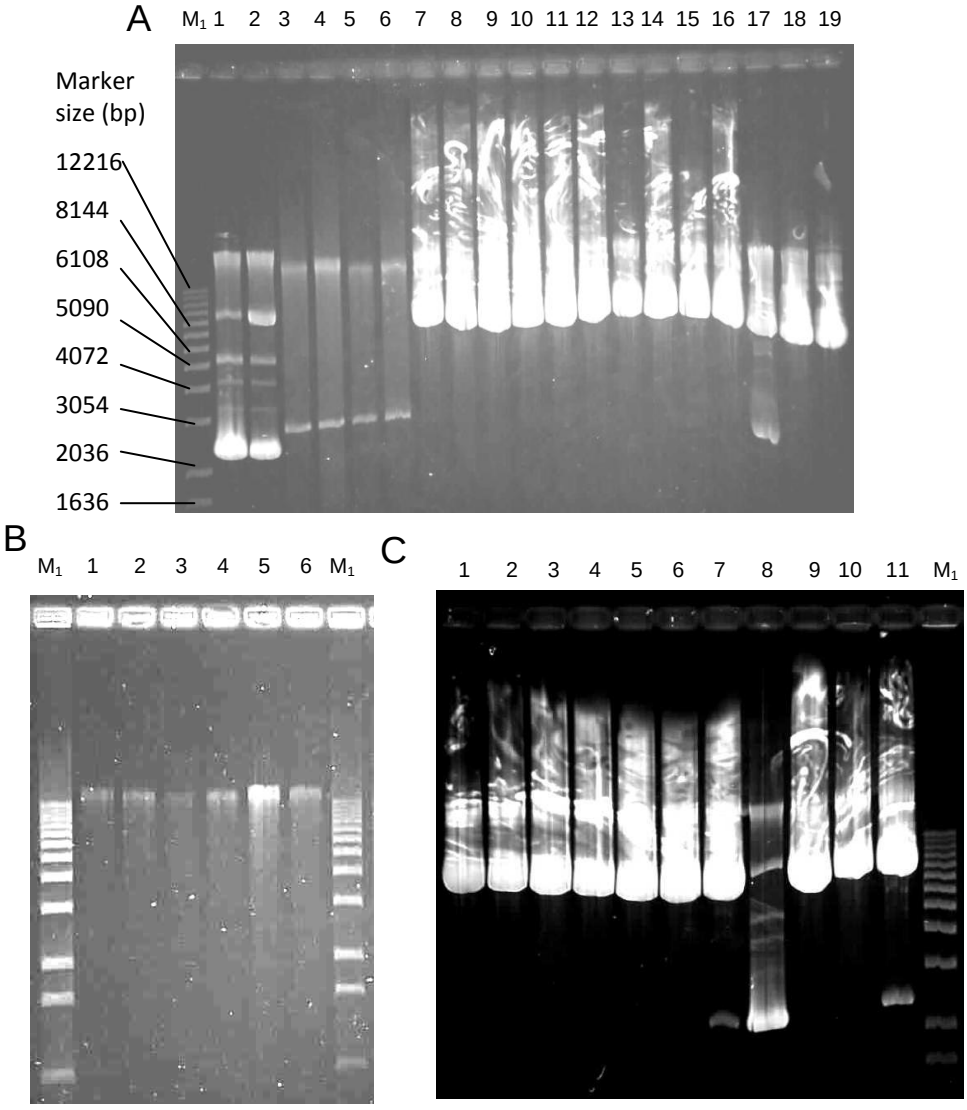


Figure 2.17: Recombinant plasmids from transformed *P. aeruginosa* ATCC 9027 cells. (A) Lanes 1-6 represent plasmid pME4510. Lanes 6-19 represent recombinant plasmids isolated from lysS-pME*lux*. (B) Lanes 1-6 represent recombinant plasmids pME*lux*. (C) Lane 1 represents ldcC-pME*lux*, lanes 2-7 represent tat-pME*lux*, lane 8 represents lys-pME*lux*, and lanes 9, 10 and 11 represent tat-pME*lux*, spc-pME*lux* and lys-pME*lux* respectively.

Following plasmid isolation, the recombinant plasmids were digested with EcoRI and SnaBI to assess the presence of the amplicons. Due to the wide range of resolution required to visualise the promoter regions (100-250bp) as well as the plasmid bands (10kb), the promoter regions were not observed upon restriction analysis. Therefore the recombinant plasmids isolated from *P. aeruginosa* were subjected to PCR amplification, results of which would show the presence of promoter inserts, if any. Figure 2.18 represents the presence of promoter regions in the recombinant plasmids isolated from *P. aeruginosa* and therefore successful introduction of *lux*-based recombinant plasmids and the creation of constitutively expressed *lux*-based bioluminescent constructs. According to Figure 2.18A, a band approximately 200bp in lanes 1-2 indicates the amplification of P_{lpp}. Bands of 220bp and 150bp in lanes 3 and 4 indicate the amplification of P_{ldcC} and P_{spc}, respectively. A band of 200bp in lanes 5-17 represents amplification of P_{lysS}. Similarly, a band of 200bp in lanes 1-7 of Figure 2.18B represents the amplification of P_{tat}. Bands of 100bp were also observed in lanes 1, 3, 5 and 7 of Figure 2.18B, indicating mis-priming of the primers.

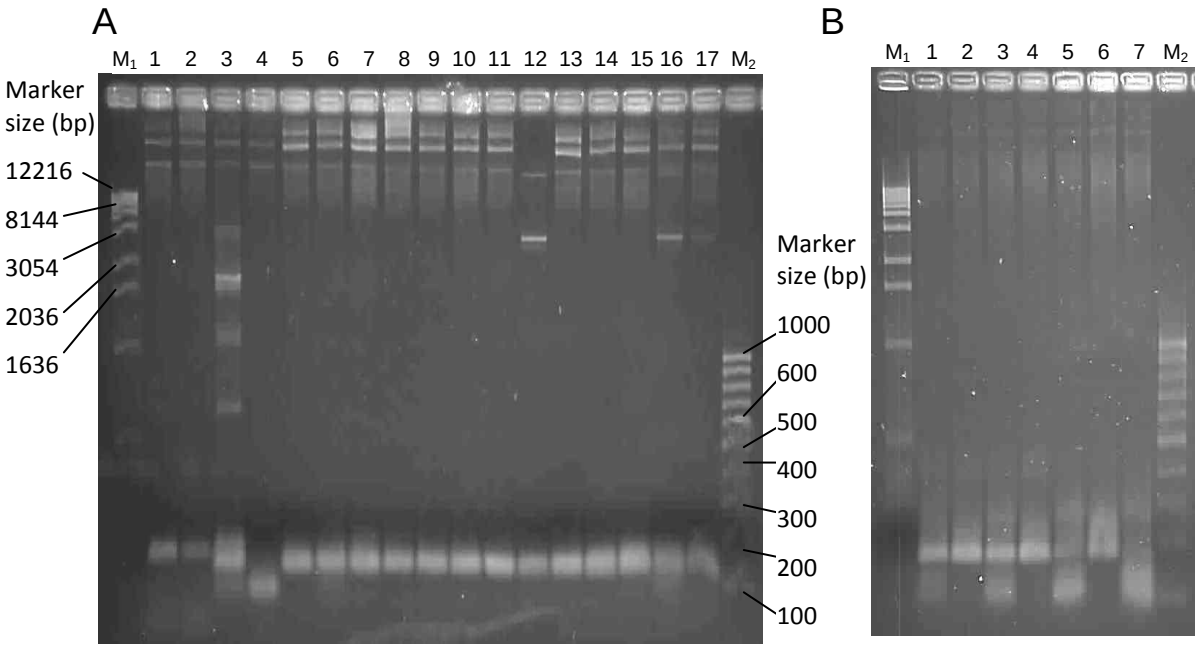


Figure 2.18: PCR reactions for P_{ldcC}, P_{lpp}, P_{tat}, P_{lysS} and P_{spc} from *P. aeruginosa* clones. (A) Lpp-pME*lux* is represented in lanes 1-2. LdcC-pME*lux* and spc-pME*lux* are represented in lanes 3 and 4 respectively. Lys-pME*lux* is represented in lanes 5-17. (B) tat-pME*lux* is represented in gel B.

2.4.18 RLU comparison between *E. coli* and *P. aeruginosa* strains

Relative production of bioluminescence for various bioluminescent prototypes in *E. coli* and *P. aeruginosa* bioluminescent strains were compared (Figure 2.19). It was observed that the strain harbouring the promoterless plasmid in *E. coli* produced greater bioluminescence compared to its counterpart in *P. aeruginosa.* However, all *P. aeruginosa* bioluminescent strains containing promoters produced bioluminescence at various magnitudes higher than their counterparts in *E. coli.* For instance, the lys-pME-*lux* was found to be 500 times more bioluminescent in *P. aeruginosa* than in *E. coli*. LdcC-pME-*lux* and spc-pME-*lux* produced less bioluminescence compared to Lys-pME-*lux*, Lpp-pME-*lux* and Tat-pME-*lux*. Different variants of the same strain also produced bioluminescence of different magnitudes; for example, three variants of Tat-pME-*lux* produced different levels of bioluminescence even though these three strains contained the P_{tat}.

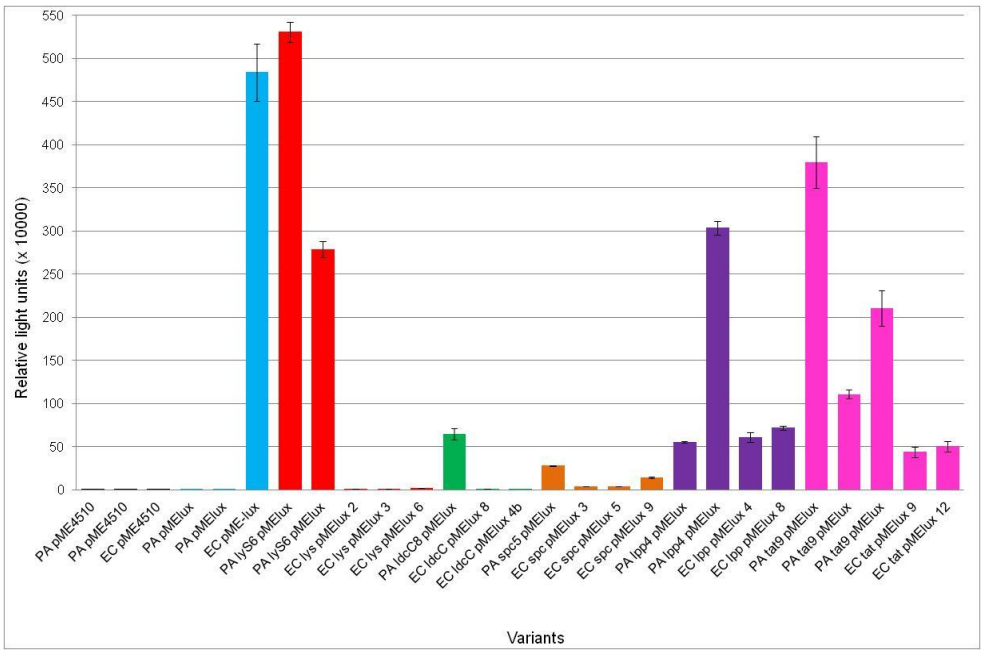


Figure 2.19: Relative light output for all *E. coli* and *P. aeruginosa* ATCC 9027 strains. Promoterless pME*lux*, lysS, ldcC, spc, lpp, tat strains. Error bars show standard deviation for n=3 samples. Bioluminescence was measured at 490nm from 1 mL of each strain grown for 24 hours.

2.4.19 Plasmid DNA sequencing of *P. aeruginosa* bioluminescent strains

All plasmid-bearing strains produced from *P. aeruginosa* were sent for DNA sequencing to analyse the nucleotide sequence for inserted promoter and *lux* cassette. All plasmids from bioluminescent strains were found to harbour the defined promoter inserts between EcoRI and SnaBI restriction sites and the *lux* cassette between EcoRI and BamHI restriction sites. DNA sequences for each of the plasmid isolates are represented in Appendix 7.

2.5 Discussion

2.5.1 Characterization of *P. aeruginosa* cells

Gram staining analysis of the bacterial cells revealed pink rod-shaped cells of approximately 2μm in length, indicating that the bacterial cells were gram negative. Moreover when these cells were grown on agar plates, a characteristic blue-green colour was observed, indicating that procyanin, a water-soluble product (Reyes, Bale, Cannon & Matsen, 1981) was produced. This pigment is produced only by *P. aeruginosa* cells (Reyes et al., 1981); hence the species of the bacterial cells was confirmed. The *P. aeruginosa* species was tested for susceptibility against several antibiotics. Out of 12 antibiotics tested, the *Pseudomonas* cells were found to be susceptible to gentamicin and polymixinB (Table 2.10). This result correlates to other studies that also found *Pseudomonas* to be susceptible to gentamicin and polymixinB (Andrews, 2009; Kronvall, 1982). This information was later used to search for a plasmid containing gentamicin-resistant or polymixinB-resistant gene and compatible with *P. aeruginosa* cells.

2.5.2 Amplification of promoter regions

The *lux* cassette in plasmid pSB417 is flanked by a number of restriction sites that include SalI and PstI (Winson et al., 1998). Similarly, plasmid pBR322 has various restriction sites in the MCS, including EcoRI and PstI. Therefore, to ligate the promoter region at the 5'-end of the *lux* cassette, an artificial SalI restriction site was tagged in the reverse primers such that the 3'-end of the promoter region would match the 5' end of the *lux* cassette by sharing a SalI restriction site. Similarly, an artificial EcoRI restriction site was tagged at the 5'-end of the forward primers such that the 5'-end of the promoter matches the 5'-end of the MCS of plasmid pBR322. Finally the 3'-end of the *lux* cassette matched the 3'-end of plasmid pBR322 by sharing a PstI restriction site.

Amplification of all promoter regions was successful after optimising various parameters for PCR. Successful amplification of all five promoter regions confirmed that the DNA template was of high purity, concentration and of working quality. It also confirmed that the dNTP mixture, magnesium chloride, Taq polymerase and reaction buffer were working. Optimizing magnesium chloride concentration in PCR reactions is vital. Magnesium chloride concentrations in the range of 1.5-4mM are required for specific amplification and prevent non-specific amplification (Oste, 1989). Multiple bands produced during the optimization process were due to non-specific annealing of primers to the template DNA. Non-specific binding of primers is also enhanced by high magnesium chloride concentrations. In most cases, this was observed when a magnesium chloride concentration of 1.5mM was used and therefore magnesium chloride concentrations were reduced.

The effect of mis-matched nucleotide bases was observed in the sequencing data, where one or two nucleotides did not match the original template sequence. For example, there was an addition of an adenine nucleotide 3bp downstream of the SnaBI restriction site in the strain labelled 'spc9-pME-*lux*' in *E. coli* (appendix 6). Each set of sequencing data also showed that the first 80 nucleotides had a high level of degeneracy. This degeneracy was due to inherent difficulties faced during sequencing reactions. However, the region where the nucleotide did not match the sequence was not vital for promoter activity; that is it was not present in the -10 and -35 regions. The nucleotide mismatch was probably due to the Taq DNA polymerase lacking proof-reading ability as it does not contain 3'-5' exonuclease activity (Eckert & Kunkel, 1991). Hence PCR reactions catalysed by this enzyme are prone to errors in incorporation of mis-matched nucleotide bases. The fidelity of Taq polymerase is 10 fold less compared to that of T4 or T7 polymerases that also lack the 3'-5' exonuclease activity. However, the conditions of the PCR reactions can greatly affect the fidelity of the Taq polymerase. Some conditions, such as decreasing magnesium chloride concentration and pH of reaction (Eckert

& Kunkel, 1990), increase fidelity. On the other hand, increasing incubation temperature from 70 to 80^{o}C (Eckert & Kunkel, 1991) and dNTP concentration from 1μM to 1mM (Eckert & Kunkel, 1991) reduces the fidelity of the enzyme. Hence, it was desirable to use low dNTP and magnesium chloride concentrations for more accurate PCR amplification.

Amplification of P_{lpp} P_{ldcC} and P_{spc} resulted in amplicons which were approximately of the correct molecular size. However, amplification of P_{tat} resulted in an amplicon that was smaller than the expected size and therefore the reaction was performed using exactly the same conditions but on an alternative non-gradient PCR machine. Using this machine, amplicons of the correct molecular size were obtained. This suggests that even a slight difference in annealing temperature can affect the amplification to obtain the correct products. Gradient PCR machines have varied temperatures across the blocks and therefore may contribute to sub-optimal temperatures. Since optimal temperatures are vital for PCR reactions, the requirement for thermal cyclers that allow stringent and accurate maintenance and control of temperature is vital. A study (Hoelzel, 1990) emphasized that a thermal cycler consisting of a metal block heater was crucial to provide uniform heating and cooling in all the wells/tubes.

With regards to the importance of temperature in PCR reactions, the denaturation temperature and the reannealing temperature are the most crucial (Bej, Mahbubani & Atlas, 1991b). In this study, the denaturation temperature was set at 94^{o}C as a standard denaturation temperature. If the denaturation temperature is too low, the double stranded DNA template will not denature to form single stranded DNA. On the other hand, the DNA is prone to damage at high temperatures especially during the denaturation step. At high temperatures cytosine is converted to uracil, which is an analogue of thymine (Eckert & Kunkel, 1991). Hence, such base substitution can lead to errors in the amplified sequences and this could be another reason for mismatches between the template DNA and amplified DNA. The reannealing temperature is equally important since it

dictates the specificity of the reaction. The reannealing temperature is partially dictated by the primer pairs and therefore needs to be optimized. Extensive work was carried out to optimize the reannealing temperature for PCR reactions for each of the promoter sequences. Low annealing temperatures results in the primers binding to non-target DNA, thereby producing multiple PCR products. If the temperature is too high, the primers cannot anneal to the target template and therefore no amplification is observed. A 2-step PCR can be performed if the expected amplification products range between 100-300bp. The only difference between a normal PCR and a two-step PCR reaction is that the extension temperature is the same as the annealing temperature in the latter (Bej, DiCesare, Haff & Atlas, 1991a).

The prerequisite of high concentrations of amplicons for subsequent restriction and ligation reactions required that the PCR reactions be scaled up to 200μL-250μL. These PCR products were then purified using the PCR purification kit and the pure products were eluted in 50μL. This step of eluting larger volumes of PCR reactions into smaller volumes brought about an intrinsic 'concentrating effect' of the PCR products during the purification step. This procedure was desirable to avoid ethanol precipitation to concentrate the amount of DNA available for subsequent reactions. Ethanol precipitation has the disadvantage of lower yield of the PCR products during the procedure and it is also time consuming.

The various promoter sequences of *P. aeruginosa* were documented and utilised successfully in this study for the first time.

2.5.3 Purification of amplicons

The PCR products were then purified by two different methods; using the gel extraction and PCR purification kits. In both cases, there seemed to be a substantial loss of the products. Hence, PCR reaction volumes were scaled up. There was no remarkable difference between the two methods

for the recovery of products as observed by absorbance readings. Hence the PCR purification method using the SIGMA ALDRICH® GenElute PCR clean up kit was subsequently used for purifying PCR products as it was a very rapid method and did not require gel extraction, exposure to UV radiation or staining with ethidium bromide. Ethidium bromide is an intercalating agent that inserts itself between bases of the DNA double helix and can cause an insertion of an extra base during DNA replication. The consequence of this is that a frameshift mutation is caused (Dale & Park, 2004). Moreover, the restriction endonuclease EcoRI digests both DNA strands at the consensus sequence 5'-GAATTC-3', producing sticky ends. However, in the presence of ethidium bromide EcoRI digests only one of the two DNA strands producing a nick in the DNA rather than a sticky end (Balagurumoorthy, Adelstein & Kassis, 2008). Exposure to UV light causes two adjacent pyrimidine bases to form covalent bonds between them. Once these covalent bonds are formed, the DNA is unable to replicate. This damage is usually repaired; however, a mutation is created as a result of such repair (Dale & Park, 2004). For these reasons, the PCR purification method was preferred; however, small aliquots of the purified products were analysed on agarose gels to confirm that the correct PCR products were obtained and purified.

The PCR products were required to be sequenced after purification to ascertain that the products were the actual promoter regions being amplified. Several attempts were made at sequencing the purified amplicons; however no sequence data were available. This was because the PCR products were unstable during the transit of the samples to the sequencing facility. The next approach therefore was to clone the amplified products into a vector before attempting to sequence them, as adviced by the sequencing facility (GATC®).

2.5.4 Plasmid isolation

The aim was to insert the *lux* cassette followed by the amplified promoter regions into the plasmid pME4510. Plasmid pME4510 contains a gentamicin resistance gene and is compatible in *Pseudomonas* (Rist & Kertesz, 1998).

Plasmid isolation was successful and, in most cases, several conformations of the plasmids were obtained, as observed by the presence of several bands and the difference in their mobilities when resolved on an agarose gel. The difference in mobilities showed that linear DNA migrates more slowly than any other conformation of DNA. Supercoiled DNA molecules migrate much faster and hence much further compared to linear DNA molecules (Berg et al., 2002; Karp, 1999). Various bands represent different conformations of the plasmid DNA molecules, such as super-coiled structures, open circular structures, linear plasmid and concatemers (Lahijani, Hulley, Soriano, Horn & Marquet, 1996). Concatemers may be obtained if the plasmid DNA was isolated whilst the bacterial cells were undergoing replication.

2.5.5 Restriction

Digestion of plasmid pSB417 with the two enzymes SalI and PstI produced only one fragment of linearised 8.5kbp plasmid, instead of the expected two fragments of molecular size 5.8kbp and 2.7kbp and therefore further investigations were performed. Single restriction digests were performed to determine whether both restriction enzymes were functional. The production of linearised fragments of 8.5kbp confirmed that both enzymes were functional. Further investigation to determine whether both these enzymes were compatible together in a double digest was performed by digesting pBR322-*lux* with these two enzymes in a double digest (Figure 2.20).

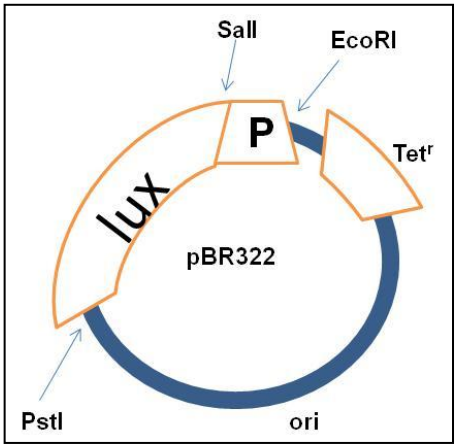


Figure 2.20: Promoter-*lux*-pBR322 plasmid (adapted from Bolivar et al., (1977). Tet^r and ori represent the tetracycline resistance gene and origin of replication, respectively. Relevant restriction endonucleases sites are also shown.

Again, the production of two fragments as expected confirmed that these two enzymes were compatible when used together in a double digest. Hence the inability to produce two fragments of plasmid pSB417 by digestion with SalI and PstI suggested that one of the restriction sites was absent. It was confirmed that the SalI and PstI restriction sites in the plasmid pSB417 were lost/mutated at either the 5'-end of the *lux* cassette or at the 3'-end of the *lux* cassette. A double restriction digest with SalI and XbaI produced two fragments of 3.8kbp and 4.7kbp (Figure 2.21), which confirmed that the restriction sites were lost/mutated at the 5'-end of the *lux* cassette.

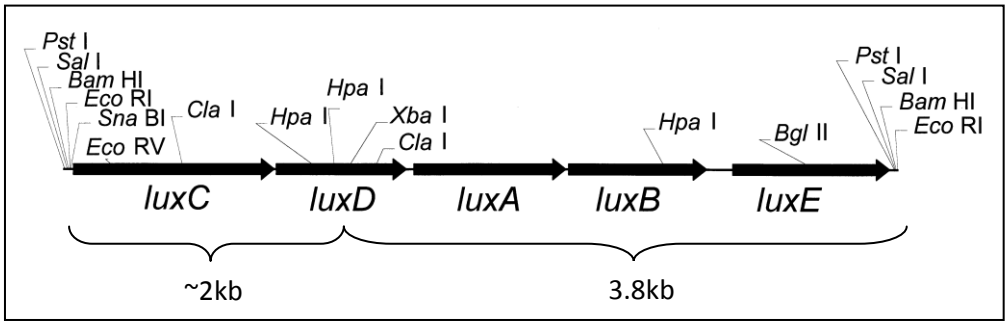


Figure 2.21: *lux*-cassette flanked by various restriction sites (Winson et al., 1998). Digestion with XbaI and SalI would be expected to produce two fragments of approximately 2kbp and 3.8kbp as shown, and also a third fragment of 2.7kbp for the remainder of the plasmid.

Failure of pSB417 digestion with SalI and PstI led to the investigation to determine whether the *lux* cassette could be excised with EcoRI and BamHI. The functionality of the enzymes in a double digest was also assessed by digesting the plasmid pBR322. Digestion of plasmid pSB417 with BamHI and EcoRI was successful, since two fragments of ~5.8kbp and 2.7kbp were produced as expected. However, absence of SalI and PstI restriction sites on plasmid pSB417 suggested that the reverse primers needed to be redesigned in order to incorporate a SnaBI restriction site rather than a SalI restriction site.

2.5.6 Formation of *lux*-bearing plasmids

The aim was to excise the *lux* cassette out of the plasmids pSB417 or 322-*lux* and insert it into the vector plasmid pME4510 (Rist & Kertesz, 1998). Double digestion of plasmid pSB417 with EcoRI and BamHI produced two fragments of the correct molecular sizes of 5.8kbp and 2.7kbp. Similarly, double digestion of plasmid 322-*lux* with EcoRI and BamHI produced two fragments of correct molecular sizes of 5.8kbp and 4kbp. The 5.8kbp band corresponded to the *lux* cassette.

Isolated plasmid pME4510 was digested with EcoRI to confirm the molecular weight of the plasmid. A linear fragment of approximately 4kb confirmed the identity of the plasmid. Transformation of plasmid pME4510 into electro-competent *E. coli* DH5α cells was successful since cells which harboured the plasmid after transformation were found to grow on LB plates containing 10μg/mL gentamicin. This further confirmed that the 4kbp plasmid pME4510 harboured the gentamicin resistance gene. Restriction digestion of this plasmid with EcoRI and BamHI and subsequent ligation with the *lux* cassette from plasmid pSB417 was successfully achieved since re-circularisation of the recombinant plasmid was observed. The resultant plasmid produced various bands of higher molecular weight, which indicated that the plasmid had re-circularised into

different conformations and that the *lux* cassette was successfully inserted into the plasmid vector. Hence, ligation was successfully achieved. Circularisation of recombinant plasmid post ligation indicated that the ligation products were actual clones and not self-ligated plasmids. Circular DNA transforms with much greater efficiency compared to linear DNA (Frerix, Geilenkirchen, Mueller, Kula & Hubbuch, 2007). The ligation mixtures were then subjected to electroporation into *E. coli* DH5α cells and screening of transformants confirmed the presence of the *lux* cassette in vector pME4510, producing pME-*lux*.

2.5.7 Insertion of promoters into promoterless plasmid vectors

Initial design of reverse primers containing the SnaBI site failed amplification on multiple occasions, even when various PCR parameters were optimised. *In-silico* studies using the OligoCalc calculator for the analysis of the newly designed primers confirmed that these primers formed hairpin loops (Kibbe, 2007) and hence could not anneal to the template DNA. Redesign of new reverse primers containing the SnaBI site lacking possibility of hairpin loop formation produced successful amplification products. The PCR products were purified and digested in double restriction digests using EcoRI and SnaBI. EcoRI digestion produces sticky ends while SnaBI digestion produces blunt ends. Hence, the digested PCR product contained a sticky EcoRI site at the 5'-end and a blunt SnaBI site at the 3'-end. This sort of digestion would allow for directional cloning where the promoter insert would ligate only in one orientation into the promoterless plasmid (Figure 2.22).

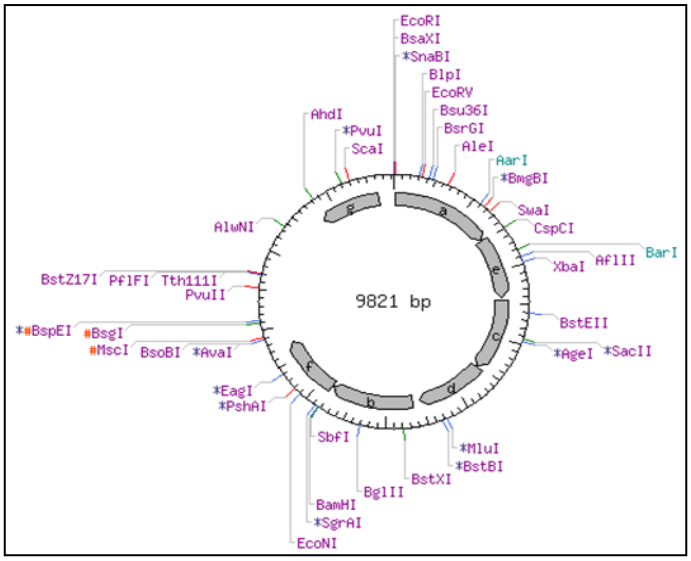


Figure 2.22: Promoterless pBR322-*lux* plasmid map (Vincze, Posfai & Roberts, 2003). The *lux* cassette, that is, *lux c*, *d*, *a*, *b*, *e* are represented by ORFs a, e, c, d and b respectively. The ampicillin resistant gene is represented by ORF g. The partial tetracycline resistant gene is represented by ORF f. * indicates that cleavage is affected by CpG methylation and # indicates that cleavage is affected by other methylation.

The promoterless plasmid pME-*lux* were digested with EcoRI and SnaBI in two separate single digests to ensure that a single fragment of linearised plasmid was obtained in both cases. This digestion was successful as linearized plasmid of ~10kbp was obtained. Subsequently the plasmid was digested with SnaBI and EcoRI in a double digest and linearized plasmid was obtained, as expected. Although a double digest under normal circumstances would produce two fragments, in this case only one visible fragment was produced because the restriction sites for both enzymes in the plasmid appear to be very close together. There is less than 20bp difference between the two restriction sites on this plasmid. Such small fragments would run off the gel when resolving larger fragments.

Following successful restriction of the PCR products and the promoterless plasmid, the two were ligated together in 24 hour ligation reactions. Ligation of promoter regions into the promoterless plasmid vectors was performed at the ratios of 3:1, 5:1, 7:1 and 10:1 of promoter inserts: plasmid vector respectively. A ratio of 10:1 for vector:insert during ligation

proved more successful because the size of the insert was relatively small (100-250bp) compared to the size of the vector (10kbp). Hence a very high concentration of the inserts was required, such that there were a significant number of inserts at the target ends compared to the number of inserts being intertwined inside the long linear DNA vector.

Bands of higher molecular sizes were obtained, indicating that new products had been formed. These products probably could be a result of cross-ligation of restricted PCR products and promoterless plasmid rather than a product of self-ligation of the plasmid only. This was because the linearised plasmid contained one sticky end and a blunt end, which would make it difficult for self-ligation. Furthermore, the ratio of 10:1 for promoter insert to plasmid vector used during ligation was considered high enough to prevent self-ligation dominance. Therefore, there was greater confidence that the ligation products were actual clones and not self-ligated plasmids. Moreover, plasmid re-circularisation post ligation gave more confidence that the ligation products were actual clones and not self-ligated plasmids. Circular DNA transforms with much greater efficiency than linear DNA (Frerix et al., 2007). Multiple bands of various molecular weights were also obtained in ligation products. These may correspond to the PCR products forming multimeric structures. However these multimeric structures would not be expected to be of very high molecular sizes; hence, these higher molecular weight bands may indicate presence of fragments of plasmid. DNA bands of the correct molecular weight were also obtained, indicating that intact plasmid was still present in the ligation reaction.

Post-ligation, the ligation mixture was heat-inactivated to denature the ligase enzyme. This was crucial for successful electroporation since active ligase inhibits electroporation. Furthermore, a high salt concentration in the ligation mixture also causes arcing during electroporation. Hence, all glassware was rinsed with deionised sterile water rather than with sterile distilled water before use. Electro-competent cells were also prepared using sterile deionised water rather than sterile distilled water. The ligase

buffer has large amounts of salt; hence, a maximum volume of 3μL of the ligation reaction was used during electroporation to prevent arcing. In the future, if higher ligation volumes are required, other ways of reducing salt content in the ligation mixture include ethanol precipitation and drop dialysis (Schlaak, Hoffmann, May & Weimann, 2005).

2.5.8 Transformation and screening

Transformation of ligation mixtures into *P. aeruginosa* was unsuccessful on several attempts. However, transformation of the same mixtures into *E. coli* was successful, as evidenced by the appearance of bacterial colonies on agar plates containing gentamicin. The plasmid pME-*lux* contains the gentamicin-resistant gene. Hence, in the presence of gentamicin, only bacterial colonies that contain the plasmids are able to survive and grow.

Transformants underwent a process of screening for clones containing either pBR322-*lux* or pME-*lux* with the promoter inserted in it. Circular plasmid DNA was obtained when plasmids were isolated from transformants during screening. Most transformants were found to contain circular plasmids of the correct molecular size. However, restriction analysis was required to confirm the correct molecular size of the plasmid and whether the plasmid harboured the promoter insert or not.

Transformation and plasmid isolation also showed that the bacterial cells were able to convert linear plasmid DNA into circular plasmid DNA. Interestingly, on screening some promoterless-*lux* transformants, it was also found that a small proportion of the transformants harboured a plasmid of molecular size much smaller than the expected ~10kbp size. Most isolated plasmids of 10kb resulted in a linear fragment of approximately 10kb, as expected during restriction analysis; however, it was found that the smaller plasmids resulted in linear DNA bands of smaller molecular weights ranging from approximately 1.6kb-4kb. Hence, it seems that the plasmid was too large to be handled by the *E. coli* strain and therefore part of it may have been excised out. The fact that the

transformants harbouring these small plasmids grew on agar plates containing ampicillin suggests that the ampicillin-resistant gene was retained. The origin of replication was also retained since these transformants were able to grow and replicate the plasmid as well; albeit a smaller plasmid. Clearly, the *lux* cassette was lost since the *lux* cassette itself is 5.8kbp in size. This sort of behaviour with plasmids losing part of their DNA sequence, has been observed previously (Clancy, Mann, Davis, & Calos, 1984), when plasmids have lost between 1-5kb of their DNA sequence during transformation. In another study performed by Maier *et al*. (2007), plasmids also lost part of their DNA sequences.

Screening the transformed colonies derived from pME-*lux*, it was revealed that some colonies harboured the specified promoter region. These strains that showed positive results for incorporation of promoter regions into the promoterless plasmid pME-*lux* were named ldcC-pME-*lux*, lysS-pME-*lux*, tat-pME-*lux*, spc-pME-*lux* and lpp-pME-*lux*. Sequence analysis of all thirteen *E. coli* strains to ascertain that the plasmids harboured the promoter regions of interest showed that DNA sequences of all promoter regions were correct. Sequencing data revealed that the amplicon inserted into the plasmids matched the specified promoter regions as expected (Appendix 6). Sequencing data also revealed that the manipulations at the three restriction sites EcoRI, BamHI and SnaBI were performed as stated in the protocols and that all three restriction sites remained functional after the manipulations, in case further manipulations were required in the future. Furthermore and more importantly, it was also revealed that the orientation of the promoter regions matched the orientation of the *lux* cassette and therefore any transcription of the *lux* cassette would be a result of the initiation by the promoters in the same orientation.

2.5.9 Transformation into *P. aeruginosa* ATCC 9027

Initial attempts to transform electro-competent *P. aeruginosa* cells failed on numerous occasions. This may have been because *P. aeruginosa*,

unlike *E. coli*, develops an alginate barrier around its membrane. Alginates are polysaccharides comprised of monomeric units of either uronic acid β-D-mannuronate (M) or uronic acid-α-L-guluronate (G) or both (Shen et al., 2006). Alginate is involved in the formation of biofilms that protect *P. aeruginosa* cells from pathogen attack and antibiotics and other invading bodies (Boyd & Chakrabarty, 1994). Introduction of DNA into *P. aeruginosa* cells containing alginates has therefore proved to be more difficult. *P. aeruginosa* cells were therefore treated with the enzyme alginate lyase prior to preparing electro-competent cells, to destroy the alginate barrier and prepare the cells to receive plasmids. Alginate lyase degrades alginate polysaccharides into their individual monomeric units by breaking the 1-4 O-glycosidic bonds (Wong, Preston & Schiller, 2000). Unfortunately, transforming electro-competent *P. aeruginosa* after alginate lyase treatment alone did not produce any transformant colonies in several attempts. Therefore, further optimisation of preparation of competent cells and the electroporation procedure was required.

Several electroporation buffers were utilised to optimize the electroporation buffer suitable for *P. aeruginosa* transformation. Various electroporation buffers found from previous studies and used for the preparation of electro-competent cells were 300mM sucrose (Smith & Iglewski, 1989), 10% glycerol (Artiguenave et al., 1997; Bloemberg, Otoole, Lugtenberg & Kolter, 1997), 25mM HEPES buffer containing 300mM glucose and 5mM calcium chloride, Ringers solution, 15% glycerol containing 1mM MOPS (Farinha & Kropinski, 1990) and finally 1mM HEPES pH 7.0, containing 300mM sucrose and 1mM magnesium chloride (Diver et al., 1990). No transformant colonies were obtained when sucrose, glycerol or HEPES buffer were used individually. However, numerous transformant colonies were obtained on selective media when the electro-competent cells were prepared in either Ringers solution or a combination of buffers containing additives such as glycerol with MOPS or sucrose with HEPES and magnesium chloride. This indicated that *P. aeruginosa* required low ionic strength buffers in the electroporation

solutions, by contrast with *E. coli*. The *P. aeruginosa* cells are larger than *E. coli* cells and therefore may undergo cell lysis at a greater rate than *E. coli* when non-ionic electroporation buffers are used. This is because the inclusion of non-ionic electroporation buffers creates an osmotic gradient when cells are electroporated and therefore *P. aeruginosa* cells are unable to recover post-electroporation. Hence, the inclusion of minimal amounts of salts such as magnesium chloride was beneficial to the recovery of *P. aeruginosa* cells after electroporation. Magnesium chloride is important in the maintenance of the structural integrity of the lipopolysaccharides (LPS) of bacterial membranes (Dennis & Sokol, 1995). Hence, the omission of magnesium chloride destabilises the LPS and this destabilisation is further enhanced during electroporation, such that *P. aeruginosa* cells are unable to recover after electroporation.

The importance of requiring salt for recovery of *P. aeruginosa* cells after electroporation was illustrated with the use of Ringers solution as an electroporation buffer. Ringers solution is a salt solution that contains sodium chloride, potassium chloride and calcium chloride. When high salt solution was used as an electroporation buffer it caused arcing during electroporation of *P. aeruginosa* cells such that the time constants of electroporation achieved were in the range 0.1-1.0 msec. However, transformation was successful when Ringers solution was used because transformant colonies were obtained upon plating the electroporated cells on selective media. In fact, all the *P. aeruginosa* cells harbouring the plasmid pME-*lux* and lpp-pME-*lux* were obtained when Ringers solution was used.

2.5.10 *Lux* expression studies in *P. aeruginosa* ATCC 9027

Investigating the ability of *Pseudomonad* promoters to initiate transcription of the *lux* cassette and produce bioluminescence showed that the bioluminescence from *E. coli* strains containing *P. aeruginosa* promoters was significantly less than that of the negative control (the promoterless *E.*

coli strain). This indicated that the *Pseudomonas* promoters were not well-recognised by the *E. coli* transcription machinery. However, the *Pseudomonas aeruginosa* bioluminescent strains containing *P. aeruginosa* promoters produced bioluminescence of several magnitudes greater than that of their *E. coli* counterparts. This was because *P. aeruginosa's* transcription machinery was able to recognise the promoter sequences that originated from *Pseudomonas* itself. A study by Rist & Kertesz (1998) also mentioned the fact that promoters originating from *P. aeruginosa* were not recognised by *E. coli*.

Transformants were also tested for bioluminescence ability and successful results were obtained where all five bioluminescent strains produced significant bioluminescence compared to the promoterless strain. LdcC and Spc exhibited the least expression of bioluminescence, while both variants of Lys exhibited similar expression of bioluminescence. Strain Lpp R4 bioluminesced three times greater than Lpp R3. Strains Tat S14 and S5 bioluminesced two-times and three-times greater than Tat S9 respectively. Variations in bioluminescence between different variants containing the same promoter suggested that plasmid copy number may vary between the variants or the variants have incorporated mutations within the promoter regions. Upon DNA sequencing, it was found that the same point mutations were present in all the plasmids; however, there were no significant mutations in the promoter regions or the whole plasmids that would lead to differences in *lux* expression. Plasmid copy number analysis also showed that plasmid copy numbers were not significantly different between different variants (as analysed in Chapter 3.4.2. Therefore, the possible reason for variation in bioluminescence between variants may be due to possible cell injury sustained during transformation that is also inherited by progeny cells.

There is very limited information about promoter structure and characterisation in *Pseudomonas aeruginosa* and therefore the data from this study will also aid in the understanding of promoters from *Pseudomonas aeruginosa*. Following successful development of working

bioluminescent constructs, the data from this study will allow quantification of bioluminescence per colony forming unit at various stages of the bacterial growth cycle.

This study reports, for the first time, the development of recombinant *E. coli* bioluminescent constructs containing constitutive *P. aeruginosa* promoters. This allows monitoring of the recognition and functionality of *Pseudomonas*-based promoters in *E. coli* (that is, whether the *E. coli* transcription machinery was able to recognize the *Pseudomonad* promoters and initiate transcription). In future, such recombinant systems may lead to promoter functional studies targeted at understanding closely the sequence requirements of promoter sequences in relation to recognition by RNA polymerase and rates of transcription initiations.

This study reports for the first time the development of functional whole cell microbial bioluminescent constructs in *Pseudomonas aeruginosa* using constitutive promoters for monitoring the global health states of *Pseudomonas* cells. These bioluminescent constructs provide the basis for use in testing efficacy of various antimicrobial agents and other compounds including novel antibiotics against *P. aeruginosa*. They may also be used for studying the strength of promotors in causing gene expression and how it can be influenced by environmental conditions.

Furthermore, the promoterless strain lacks any promoters upstream of the *lux* cassette and therefore may be used as a screening tool for further evaluation of other DNA sequences that may not currently be recognised as promoters.

2.6 Conclusion

Insertion of the promoter-*lux* insert into the host plasmid pME4510 and subsequent transformation into *E. coli* DH5α cells was successfully achieved. The transfer of plasmids from *E. coli* to *P. aeruginosa* was more challenging, since transformation into *P. aeruginosa* failed on numerous occasions. However, optimising the electroporation buffer to suit *Pseudomonas* was vital in achieving successful results with transforming plasmids. Electroporation of plasmids into *Pseudomonas* was more successful when electroporation buffers included salts that aid in post-electroporation recovery of the cell membranes. Transformants of *Pseudomonas aeruginosa* ATCC 9027 were successfully generated and confirmed by plasmid extractions from transformants and subjecting them to restriction digestion analysis, PCR amplification of promoter regions and DNA sequence analysis. Transformants were also tested for bioluminescence ability and successful results were obtained where all five bioluminescent strains produced significant bioluminescence compared to the promoterless strain. LdcC and Spc exhibited the least expression of bioluminescence, while both variants of Lys exhibited similar expression of bioluminescence. Strain Lpp R4 bioluminesced three times greater than Lpp R3. Strains Tat S14 and S5 bioluminesced two-times and three-times greater than Tat S9 respectively.

Chapter 3: Validation of *P. aeruginosa* bioluminescent strains

3.1 Introduction

3.1.1 Method validation

Method validation is defined as a process of determining whether a particular method is appropriate for its proposed use (Green, 1996; Gonzalez & Herrador, 2007). Validating a method therefore follows method development and optimisation. Immediately following method development, the operational limits and performance features for new methods need to be ascertained. This process forms part of the primary validation. Following successful primary validation, the new method is utilised to gather data and collect evidence that the new method meets the criteria that are set up during primary validation (ISO, 2000). Primary validation is usually conducted in the same laboratory as method development and aims to define certain criteria based on the method's performance in the laboratory. Validation of microbiological methods may be qualitative as well as quantitative. Parameters that need to be determined for new microbiological method validation are accuracy, precision, sensitivity, limit of detection and limit of quantification, linearity, range and equivalence.

Accuracy is defined as closeness of the true results obtained by the test method to those of the current method (ISO, 1994a, 1994b; Kramer et al., 2008; Krouse, 2005; PDA, 2000). Accuracy, which is expressed as percentage recovery of microorganisms following serial dilution, is limited to between 30 and 300 colonies for any particular dilution using the plate count method. Any new method is expected to perform better or in a similar manner to the traditional plate count method. The acceptance criterion is therefore set at 70% recovery.

Precision is defined as the level of agreement between individual test results when the method is applied to samples in replicate (Kramer et al., 2008; PDA, 2000). Precision is usually expressed as standard deviation or percentage relative standard deviation (% RSD) and may be divided into reproducibility and repeatability. The difference between repeatability and

reproducibility is that the former refers to validation conducted in the same laboratory by the same analyst using the same equipment, while the latter refers to the validation performed in the same laboratory but under varying conditions (ISO, 1994b, 1994c; PDA, 2000). The acceptance criterion is set between 15-30% RSD.

Sensitivity is defined as the minimum quantitative measurement of bioluminescence units that can be detected by a certain number of micro-organisms (Parshionikar et al., 2009) and is usually determined by testing serial dilutions of known microbial number in replicate.

Limit of detection (LOD) is defined as the detection of smallest and greatest number of microorganisms in a sample (Kramer et al., 2008; Krouse, 2005; PDA, 2000). Hence, the LOD only establishes qualitatively the presence or absence of microorganisms. It is therefore vital that this determination be performed in replicate. Limit of quantification (LOQ) is defined as the accurate and precise quantification of the least number of microorganisms in a sample (Krouse, 2005; PDA, 2000). LOQ determination should be performed in replicate at five different points, at least, across the range.

Linearity is defined as the relationship in which the results from the new method are directly proportional to the number of microorganisms present in a sample within the range (Kramer et al., 2008; Krouse, 2005; PDA, 2000). Determination of linearity should be performed in replicate at five different points, at least, across the range. Correlation coefficient (r) gives a measure of the proportionality and should lie in between 0.8 and 1.0 for a good linear relationship. In the case of microbial growth and gene expression, log-linear calibration curves are usually established.

Range is defined as the interval between the smallest and greatest number of microorganisms that can be quantified with precision, accuracy and linearity (Krouse, 2005; PDA, 2000). Validation of this range should include samples at both of the extremes of the range as well as within the range. Equivalence is defined as a measure of the similarity between the

results obtained from the new method and the traditional method (PDA, 2000). Due to the variation in sampling microorganisms, both methods should be done in parallel. Equivalence is measured by performing two-way analysis of variance (ANOVA) and a student t-test; and determining the significant difference between the two methods (Kramer et al., 2008).

Finally, it is imperative to remember that absolute quantification of the true number of microorganisms may be impossible. The basic reason for this is that microbiological methods are based on an assumption that microorganisms are dispersed equally through normal distribution in cultures. However, this is not the case. In fact, the skewed distribution leads to erroneous interpretation of statistical data and therefore microbial counts are transformed to a $\log_{10}$ –scale to convert the data into a normal distribution (Krouse, 2005; Parshionikar et al., 2009).

3.1.2 Plasmid copy number

Protein productivity from recombinant plasmids in bacteria is dependent on number, structure and stability of plasmids. Plasmids must be able to exist stably inside their host cell with minimum effects on metabolic and genetic load (del Solar & Espinosa, 2000). The first stage of the plasmid life cycle as it enters a host is called establishment. During this stage, the plasmid must be able to divide rapidly prior to host cell division. The next stage is when the plasmid is maintained during steady state growth of the bacterial host. On average, plasmids replicate once per cell replication. Hence the plasmid numbers remain virtually the same and under tight control of negative control systems (del Solar & Espinosa, 2000; Nordstrom & Dasgupta, 2006).

Plasmid stability and maintenance has been a dilemma for many years, although in laboratory settings, this is easily achieved by antibiotic resistance genes on the plasmid and the addition of antibiotics to culture media. Therefore, to understand recombinant protein productivity better, it is essential to understand plasmid stability and determine plasmid copy

number. These features also allow better understanding of cell replication in the presence of plasmid DNA. Plasmid copy number is defined as the number of plasmid copies present per bacterial cell or per bacterial chromosome (Coronado, Vazquez, Cebolla & Palomares, 1994; Lee, Kim, Shin & Hwang, 2006a; Lee, Ow & Oh, 2006b) and can vary between a few to hundreds per cell (Nordstrom & Dasgupta, 2006). It has also been shown that bacterial cells in biofilms have increased numbers of plasmid copies compared to bacterial cells in planktonic states (Cook & Dunny, 2013).

Generally, plasmid copy number can be determined using either indirect or direct methods. Indirect methods include measurement of recombinant reporter protein activity normalised to number of bacterial cells. However, more direct methods include chromosomal and plasmid DNA extraction and quantification by DNA hybridisation, gel electrophoresis and high performance liquid chromatography (Lee et al., 2006a; Lee et al., 2006b; Skulj et al., 2008). It must be appreciated that these methods are laborious, time-consuming, inaccurate and inefficient. Gel electrophoresis further proves to have low sensitivity. Furthermore, plasmid profiles obtained using gel electrophoresis indicate several isoforms; supercoiled, open-circular, linear, multimers. With the advent of conventional PCR, sensitivity of plasmid copy number determination has increased and large numbers of samples can be processed. However the disadvantage with conventional PCR is the subsequent quantification of DNA by gel electrophoresis.

Quantitative PCR (qPCR) is now widely used for DNA quantification and identification because it provides high sensitivity and improved speed, it is less laborious and gives reliable results in ‘real-time’ mode that does not require separation of plasmid DNA from chromosomal DNA (Lee et al., 2006a; Lee et al., 2006b; Skulj et al., 2008). Quantitative PCR is similar to conventional PCR; however, amplification of DNA is measured by fluorescence detection during amplification instead of end-point detection. Conventional PCR determines the amount of DNA accumulated at the end

point whereas quantitative PCR establishes the point when amplification is first detected (Huggett & Bustin, 2011). Fluorescent detection is based on the ability of non-specific fluorescent dyes such as Sybr® Green to bind to double stranded DNA in its minor groove (Huggett & Bustin, 2011; Li, Xi, Yang, Hawkins & Schubart, 2003). The dye fluoresces upon double-stranded DNA binding; hence as the amount of amplified product increases after each PCR cycle, the fluorescence intensity increases. It is this increase in fluorescence that is recorded with each cycle. The cycle number at which the fluorescence intensity is detected above a background threshold is referred to as threshold cycle (C_t) or crossing point (Cp) (Wong & Medrano, 2005) as exemplified in Figure 3.1.

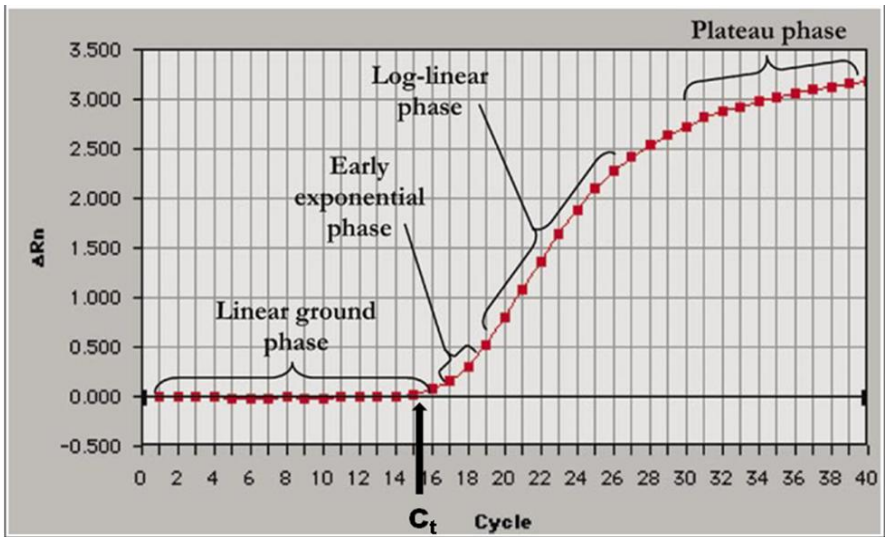


Figure 3.1: Phases of quantitative PCR quantification. Adapted from Wong & Medrano (2005). Linear ground, early exponential, log-linear and plateau phases are shown. C_t values are measured at the first observable signal above baseline.

The signal output from quantitative PCR can be divided into four phases (Figure 3.2) (Wong & Medrano, 2005). The linear ground phase is the first phase of PCR and indicates that there is no increase in fluorescence. Threshold background fluorescence is determined at this stage. The early exponential phase is the second phase where amplification has just begun. It is used to calculate the C_t value since the fluorescence level is

greater than the threshold value. The third phase is the log-linear phase when amplification is increasing at an exponential rate. This means that PCR products double with each cycle increment. The final phase is the plateau phase when the reagent concentrations become limiting and therefore there is no significant amplification or fluorescence increase.

The C_t value is vital in quantifying the number of plasmids because it is inversely proportional to the copy number (Huggett & Bustin, 2011; Wong & Medrano, 2005), meaning that the copy number is large at low C_t values and small at high C_t values. C_t values can be affected by PCR efficiency. Theoretically, amplification efficiency is regarded as 1 when fluorescence doubles with every cycle. However, in reality this may not be the case. Hence, efficiencies between 90 and 110%, calculated from the slope of a calibration curve being between -3.3 and -3.6, are acceptable (Applied Biosystems; Fraga, Meulia & Fenster, 2008; Wong & Medrano, 2005).

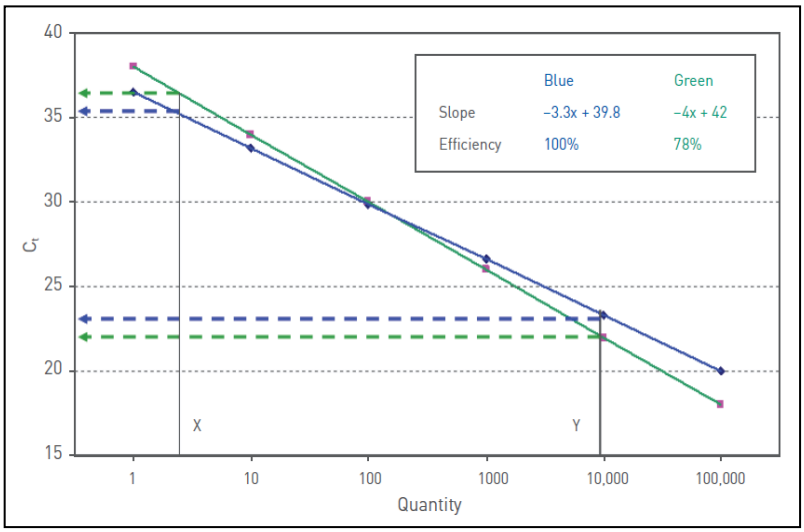


Figure 3.2: Effect of slope on PCR efficiency. C_t refers to the threshold number of cycles at which product amplification is first detected. Quantity refers to the concentration of intial DNA template present in the reaction mix in a log scale (Applied Biosystems).

The correlation coefficient (r) of a standard curve also provides a good indicator of PCR efficiency (Applied Biosystems). Generally, r-values

greater than 0.99 indicate a strong correlation between the C_t value and DNA concentration.

The high specificity of quantitative PCR is emphasized in dissociation curve analysis (Huggett & Bustin, 2011; Lee et al., 2006a; Lee et al., 2006b; Li et al., 2003; Wong & Medrano, 2005), which follows at the end of the amplification process. Also known as melt curve analysis, the double stranded DNA product post-amplification undergoes a melting process by constant increment in temperature. At a particular temperature called the melting temperature (Tm), double stranded DNA melts and converts to single stranded DNA and the fluorophore unbinds as the DNA melts. As a result, the fluorescence intensity decreases drastically. Different PCR products have different Tm; therefore, any non-specific products formed during the amplification process can be detected from the melt curve analysis (Figure 3.3).

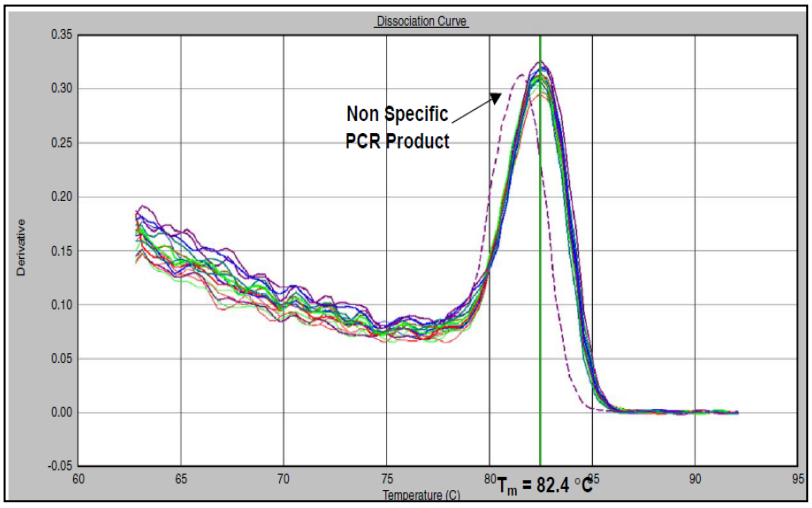


Figure 3.3: Melt curve profile for a typical real-time PCR amplification. Adapted from Lee *et al.* (2006b). The temperature at which single-stranded PCR products are observed is the T_m temperature that results into peaks. Peaks at temperatures different from the T_m are non-specific PCR prodcuts (dotted line) that result from mis-priming.

3.2 Aim and objectives

The aim of this work was to validate all the bioluminescent strains using the bioluminescence method to determine whether the newly created bioluminescent constructs were functional and stable.

The objectives of these studies were:

- To assess the parameters such as linear range, accuracy, precision, equivalence, limit of detection and limit of quantification as set out in various documentations (ISO, 1994a, 1994b, 1994c, 2000; PDA, 2000) using the bioluminescent method.
- To assess plasmid copy numbers by quantitative PCR.

3.3 Materials and methods

3.3.1 Bacterial strains

The *Pseudomonas* bacterial strains used for validation assays are listed in section 2.3.1. All bioluminescent strains and negative controls from the master plates were inoculated onto working LB agar plates containing 10μg/mL gentamicin (except for WT that was inoculated into LB without antibiotic) and incubated for 24 hours at 32^{o}C. Upon growth, single isolated colonies of all strains from the secondary plate were then inoculated into culture flasks containing 5 mL of LB broth and 10μg/mL gentamicin (except for WT that was inoculated into LB without antibiotic) and incubated for 24 hours at 32^{o}C in an incubator shaking at 250rpm.

3.3.2 Promoter strength determination

For determination of promoter strength, isolated colonies of all variants of the *P. aeruginosa* ATCC 9027 bioluminescent strains and negative controls were grown as described in section 3.3.1. Cultures were then grown by inoculating 20 μL of the starter cultures into culture flasks containing 9 mL of LB broth and 10μg/mL gentamicin (except for WT that was inoculated into LB without antibiotic) and incubating at 32^{o}C in a shaking incubator shaking at 250rpm for 24 hrs.

An aliquot (0.5 mL) was withdrawn from each culture flask and serially diluted into 4.5 mL of buffered peptone water (Oxoid Ltd). An aliquot (1 mL) of each dilution was transferred into a culture tube aseptically for RLU determination as described in section 2.3.15.

For CFU determination, aliquots (0.1 mL) of each dilution were also spread aseptically on LB agar plates containing 10 μg/mL gentamicin (except for WT that was spread on LB agar plates lacking antibiotic) and incubated at 32^{o}C for 48 hrs.

3.3.3 Linearity and standardisation of inocula

The objectives of this experiment were to determine the linear range of the assay, standardise the inocula for preservative efficacy tests and to determine the dilution factor to be used for accurate RLU measurements.

Strains were grown as described in section 3.3.1. Following incubation, all samples were serially diluted 10 fold seven times in buffered peptone water. Aliquots (1 mL) of undiluted and diluted cultures were transferred into culture tubes aseptically for bioluminescence measurements. Relative light units (RLU) were measured using the Celsis Advance Luminometer as described in section 2.3.15. Aliquots (100 μL) of undiluted and diluted cultures were plated as described in section 3.3.2.

3.3.4 Accuracy testing

The objective of this experiment was to determine the accuracy of the bioluminescent method. Strains were grown as described in section 3.3.1. Following incubation, all samples were serially diluted 10-fold in buffered peptone water. For undiluted, 10-fold and 100-fold diluted samples, further dilutions were performed, in triplicate, with buffered peptone water as indicated in Table 3.1.

Table 3.1: Percentage dilution range for accuracy testing

Cells (%)	*Volume of cell culture (μL)*	*Volume of diluent (μL)*
0	0	1000
10	100	900
25	250	750
50	500	500
75	750	250
90	900	100
100	1000	0

Aliquots (1 mL) of each dilution were transferred into culture tubes aseptically for bio-luminescence measurements as described in section 2.3.15. Aliquots (100 μL) of each undiluted and each diluted culture were transferred onto TSB Agar plates as described in section 3.3.2.

Mean RLU values were calculated by summing the RLU values in a group and then dividing the sum by the number of RLU values. Standard deviation was calculated by using the STDEV function in MS EXCEL for a group of RLU values. Recovery (%) was calculated for each dilution for all strains.

Percentage Recovery was calculated as follows:

$$\left[\frac{\text{Mean RLU at given number of cells}}{\text{Mean RLU at 100\% cells}}\right] \Big/ \text{Expected dilution factor} \times 100$$

Equation 1: Percentage recovery calculation

3.3.5 Precision testing

The objective of this experiment was to determine the precision of the bioluminescent method. Strains were grown as described in section 3.3.1. Following incubation, all samples were serially diluted 10-fold in buffered peptone water. This procedure was repeated 10 times. Aliquots (1 mL) of each dilution were transferred into culture tubes aseptically for bio-luminescence measurements as described in section 2.3.15. Aliquots (100 μL) of undiluted and diluted cultures were transferred onto TSB agar plates as described in section 3.3.2.

Mean and standard deviation values were calculated for each dilution as described in section 3.3.5. Percent relative standard deviations (%RSD) for each dilution were calculated as follows:

$$\frac{\text{Standard deviation of RLU}}{\text{Mean RLU}} \times 100$$

Equation 2: Relative standard deviation calculation

3.3.6 Data analysis

Statistical analysis was done using SPSS for Windows. The data were checked for normality (Shapiro-Wilk test) and constant variance. Pearson's correlations between bioluminescence reading and plate count were performed at a significance level of 95% for data obtained from linearity and accuracy studies. One-way ANOVA and an F-test at a significance level of 95% were performed on precision data to determine the variability between the bioluminescence and plate counting methods. Two-way analysis of variance (ANOVA) at a significance level of 95% was performed between bioluminescence and plate count data to determine the equivalence between both methods.

3.3.7 Plasmid copy number

All strains were grown as described in section 3.3.1. An isolated colony of each strain was inoculated into 100 mL LB containing 10 μg/mL gentamicin (except for WT that was inoculated into LB without antibiotic) and incubated at 32^{o}C and 150 rpm shaking conditions for 28 days. Samples were taken from each of the flasks at 6 hours and 24 hours after inoculation, followed by sampling at 7 days, 14 days, 21 days and 28 days after inoculation.

3.3.7.1 DNA extraction

For calibration of standard curves, genomic DNA was isolated according to the manufacturer's protocol using the GenElute Bacterial Genomic DNA Kit purchased from Sigma-Aldrich®. Plasmid DNA was extracted as

described in section 2.3.9. For samples of unknown DNA concentration, total DNA was extracted by using the QIAamp® DNA Mini and Blood Mini Kit purchased from QIAGEN according to the manufacturer's protocol. All eluates containing DNA were either stored at -20^{o}C or used for further analysis.

3.3.7.2 Quantitative Polymerase Chain Reaction (qPCR)

For the determination of plasmid copy number, specific forward and reverse oligonucleotide primers were designed for the *DxS* gene, a housekeeping gene on the chromosome, and the gentamicin gene on the plasmid (Table 3.2) using PRIMER 3 software. The melting temperatures of the primers were calculated using 'BioMath - Tm Calculations for Oligos' software (Promega Corporation). Oligonucleotide primers were then constructed by Invitrogen Ltd.

Table 3.2: Forward and reverse primers for amplification of DxS and gentamicin.

Primer Name [a]	**Primer**	**Primer length (bases)** [b]	**% GC content**[c]	**Tm (^{o}C)** [d]
DxS F	5'-CATCAGGTAAACTGCTGGCCTCTC-3'	24	54	54
DxS R	5'-CCGACGGTATACAGCAGGTACTGG-3'	24	58	56
Gent F	5'-GCACTGCATAATTCTCTTACTGTCA-3'	25	40	49
Gent R	5'-GCTCTCTATACAAAGTTGGGCATAC-3'	25	44	51

[a] F denotes forward primer and R denotes reverse primer. The primer length[b] is between 30-40bp, % GC content[c] is around 50% and the melting temperature[d] for each primer is between 60-70^{o}C.

Stock and working primer suspensions were prepared as described in section 2.3.6. The initial qPCR reaction mixtures were prepared in sterile PCR tubes (Table 3.3). The Platinum SYBR® Green qPCR SuperMix-UDG Kit was purchased from Invitrogen Ltd. The following reagents were added making up a total volume of 50 μL.

Table 3.3: Various components of a qPCR reaction.

Reagent	**Volume (μL)**	
	DxS gene	**Gentamicin gene**
Sybr Green Mix	25	25
Bovine Serum Albumin	1	1
10μM DxS Forward primer	1	
10μM DxS Reverse primer	1	
10μM Gent Forward primer		1
10μM Gent Reverse primer		1
Genomic DNA template	1	
Plasmid DNA template		1
Sterile water	21	21
Total volume	50	50

Volumes for each reagent at stated concentrations for qPCR of *dxS* and *gent* genes.

The reaction tubes were placed in the quantitative PCR thermocycler (DNA Engine Peltier Thermal cycler with Chromo4 Real-time PCR detector from Biorad). The cycling conditions for the qPCR reactions were optimised from another study (Lee et al., 2006) (Table 3.4).

Table 3.4: Cycling parameters for quantitative PCR reactions

Steps		**Temperature (^{o}C)**	**Time (minutes)**
1	Start	50	2
2	Initial Heat Denaturation	96	10
3	Heat Denaturation Primer annealing Primer extension	95 Gradient temperature 72	1 1 1
4	Extension	72	10
5	Plate read		
6	Melt curve	40-95 (read at every 0.2^{o}C)	2 sec at each temperature

30 cycles

Several qPCR reactions were performed at a range of annealing temperatures between 45^{o}C – 60^{o}C and 46^{o}C – 56^{o}C for gentamicin and DxS gene amplification, respectively. Upon optimisation, the PCR reactions were performed at 60^{o}C and 46^{o}C, respectively. A PCR reaction

lacking the DNA template was also prepared as a negative control. Following PCR, 10 μl of the products from each reaction tube were resolved on a standard 1.5% agarose gel prepared as specified in section 2.3.8. A 100bp standard DNA marker was also loaded on the gel for verification of PCR product sizes.

3.3.7.3 Melt curve generation

Melting point analysis was performed to differentiate target PCR products from misprimed products. After completion of PCR, temperatures were decreased to 40^{o}C and then increased at a rate of 0.2^{o}C every two seconds until they reached 95^{o}C. The fluorescent signal at each temperature increment was measured and a melt curve was constructed.

3.3.7.4 Analysis

Standard calibration curves were generated for genomic and plasmid DNA. Various concentrations of genomic and plasmid DNA were used to determine the C_t values and construct calibration curves. Concentrations were then extrapolated from respective C_t values for unknown samples and the corresponding concentrations of genomic DNA and plasmid DNA were determined. Plasmid copy numbers were calculated using the formula:

$$PCN_{\text{per genome}} = \frac{\text{genomic DNA size (bp)}}{\text{plasmid DNA size (bp)}} \times \frac{\text{Amount of plasmid DNA (}\mu\text{g)}}{\text{Amount of genomic DNA (}\mu\text{g)}}$$

Equation 3: Plasmid copy number calculation (Lee et al., 2006).

Pearson's correlations were calculated between plasmid copy number and time at a significance level of 95% using SPSS.

3.4 Results

3.4.1 Validation

The bioluminescent method was validated against the traditional method of plate counting using several validation parameters.

3.4.1.1 Linearity studies for all strains

Relative production of bioluminescence was compared to CFU count for all *P. aeruginosa* bioluminescent strains to determine the linear range of the bioluminescent assay (Figure 3.4). Relative light unit (RLU) measurements were determined for each serial dilution for all strains. Wild-type, pME4510 and pME*lux* provided negative controls with respect to RLU measurements and produced bioluminescence readings of 10^2 RLU. Sigmoidal relationaships between RLU and CFU were obtained for all bioluminescent strains. Statistical analysis of the data using normality test (Shapiro-Wilk) and constant variance test showed that the P-values and W-values for all bioluminescent strains were greater than 0.05. Therefore all bioluminescent strains passed the normality and the constant variance tests (Appendix 8).

A horizontal relationship between RLU and CFU count in the range from 1-10^4 CFU counts was observed. When CFU count decreased below 10^3 CFU/mL, bioluminescence remained constant at baseline and was undistinguishable from the negative controls. This indicated that the lower limit of detection for RLU measurements was limiting measurement of bioluminescence for those samples. RLU reading increased proportionately with increase in CFU count from 10^4 to 10^8 CFU/mL. Furthermore, good correlations were observed for RLU per CFU count from 10^4 to 10^7 CFU/mL. However, detection of bioluminescence for all bioluminescent constructs was less than expected for 10^8 CFU/mL as compared to 10^7 CFU/mL as shown by a non-linear relationship when

CFU count was greater than 10^7 CFU/mL. Consistent results were observed for all bioluminescent strains compared to the negative controls that harboured either the plasmid only or the promoterless plasmid. In all cases, ldcC and spc gave the lowest light output and lys gave the highest light output per CFU.

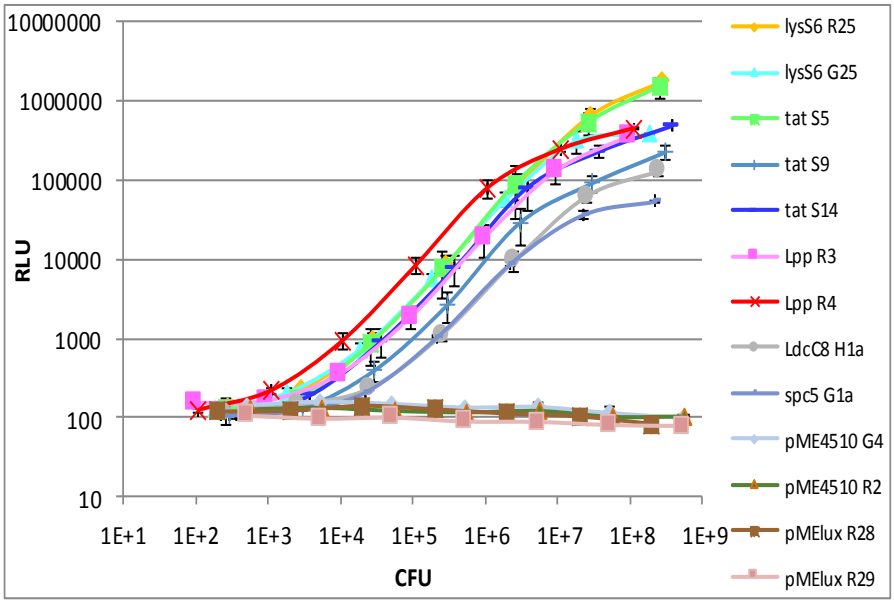


Figure 3.4: Relationship between RLU and CFU for all *P. aeruginosa* ATCC 9027 bioluminescent strains. Bioluminescence and CFU counts were measured from 1 mL, in triplicate, for all strains grown for 24 hours. Error bars indicate standard deviation from n=3.

Following from Figure 3.4, the relationships between RLU and CFU for all strains were quantified and the correlations indicated in Table 3.5. Strong correlations were obtained for the range 10^3 – 10^7 CFU/mL. This was the linear range of the graph (Figure 3.4). Correlations were slightly less for the wider range 10^3 – 10^8 CFU/mL. Correlations were significant at $P<0.01$ for all bioluminescent constructs within the range 10^3 – 10^7 CFU/mL. Correlations were significant for most strains at $P<0.01$ for the range 10^3 – 10^8 CFU/mL; however, the significance was $P<0.05$ for lppR4 and spc. Correlations were significant ($P<0.01$) but not as good for the full range of serial dilutions tested for all bioluminescent strains except lysG25. This indicated that it was not advisable to examine undiluted cultures for RLU measurements.

Table 3.5: Correlation between RLU and CFU for all strains

Strain	10^2 to 10^8 Range		10^3 to 10^8 Range		10^3 to 10^7 Range	
	Correlation	Sig.	Correlation	Sig.	Correlation	Sig.
lysS6 R25	0.965**	0.000	0.965**	0.002	0.999**	0.000
lysS6 G25	0.787*	0.020	0.773	0.071	0.997**	0.000
tat H5	0.970**	0.000	0.970**	0.001	0.998**	0.000
tat H9	0.951**	0.000	0.951**	0.003	0.977**	0.004
tat H14	0.928**	0.001	0.927**	0.008	0.970**	0.006
Lpp R3	0.966**	0.000	0.965**	0.002	0.999**	0.000
Lpp R4	0.902**	0.002	0.900*	0.015	0.974**	0.005
LdcC8 H1a	0.932**	0.001	0.930**	0.007	0.999**	0.000
spc5 G1a	0.856**	0.007	0.850*	0.032	0.990**	0.001
pME4510 G4	-0.790*	0.020	-0.780	0.067	-0.833	0.080
pME4510 R2	-0.628	0.096	-0.600	0.208	-0.852	0.067
pME*lux* R28	-0.814*	0.014	-0.811	0.050	-0.747	0.147
pME*lux* R29	-0.628	0.095	-0.659	0.155	-0.799	0.105

** Correlation significant at 0.01 level. * Correlation significant at 0.05 level.

Relative production of bioluminescence per CFU count was determined at a range of serial dilutions for all *P. aeruginosa* bioluminescent strains (Figure 3.5) after 24 hours of growth. Consistent results were observed for 10-fold and 100-fold dilutions and hence these two dilutions were chosen to depict the light output per unit CFU for all the bioluminescent strains with an objective of determining relative strength of five different promoters. It was observed that both variants of lpp (lpp R3 and lpp R4) produced similar RLU readings per CFU. Similarly, both strains of lys produced similar RLU readings per CFU. Strain tat H5 produced three-fold and two-fold higher bioluminescence than its variants tat H9 and tat H14, respectively. Strains harbouring ldcC and spc gave the lowest light output of the five promoters. Beyond 10^{-5} dilutions the log RLU: log CFU increased with increase in dilution factor and therefore correlations were poor. All negative controls were observed to produce bioluminescence less than the lower limit of detection.

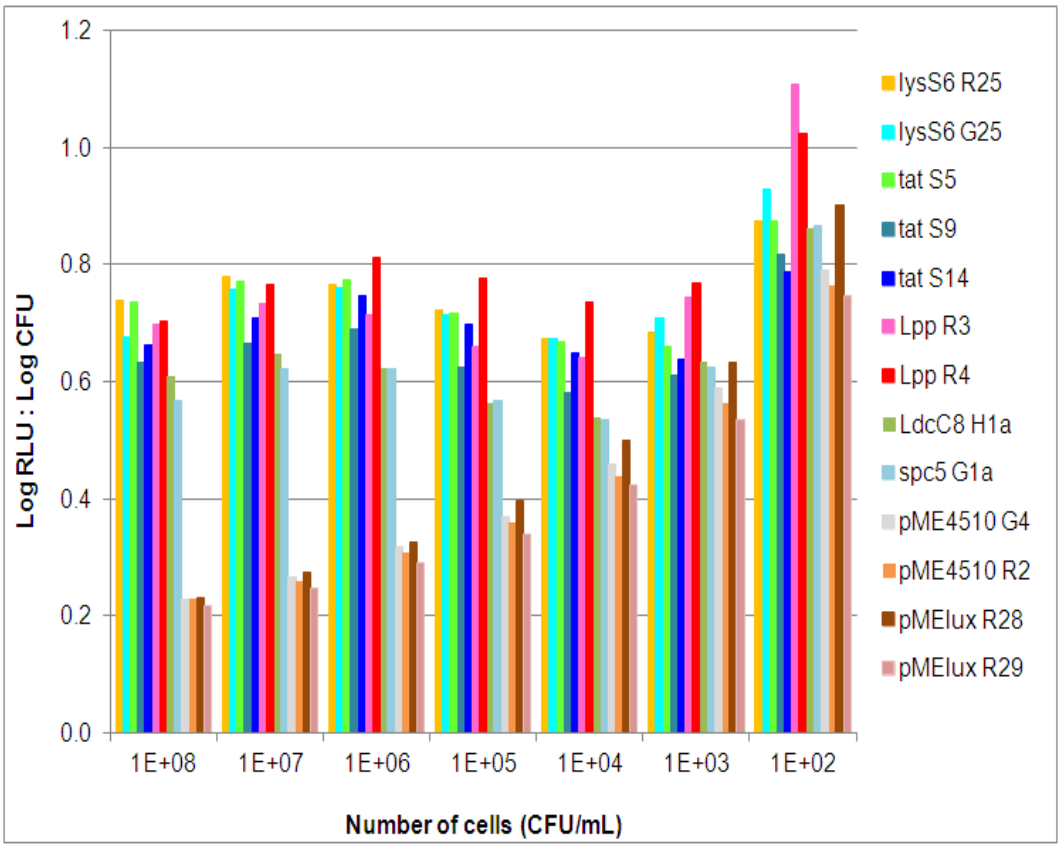


Figure 3.5: Consistent relationship between Log RLU:Log CFU at various serial dilutions for all *P. aeruginosa* ATCC 9027 bioluminescent strains. pME4510 and pME*lux* are control strains that do not bioluminesce. Bioluminescence was measured at 490nm from 1 mL of each strain that was serially diluted 1:10.

3.4.1.2 Accuracy

The next step of the validation process was to determine the accuracy of the bioluminescent method compared to the traditional plate count method. It was elucidated whether the acceptance criteria of 100% ± 30% was achieved. The percentage recovery of bioluminescence from undiluted cultures and serially diluted cultures were tested. As shown in Table 3.6, the percentage recoveries of undiluted cultures (10^8 CFU/mL) for all bioluminescent constructs were outside the acceptance criteria of 70%. However, 10-fold and 100-fold serially diluted cultures produced recoveries of between 70% and 130%. These dilutions for all strains therefore passed the acceptance criteria.

Furthermore, it was determined whether bioluminescence reduced with each diluted suspension within the ten-fold serial dilutions. Pearson's correlations indicated that there was a very strong relationship between bioluminescence and CFU count within the diluted suspensions for all serial dilutions ($r > 0.8$) except the undiluted cultures of ldcC ($r = 0.45$). Strong correlations were also found to be statistically significant at $P<0.01$ in most cases except undiluted cultures of spc, which was significant at $P<0.05$.

Table 3.6: Accuracy analysis and Pearson's correlations.

Strain	Number of cells (CFU/mL)	Recovery (%) at indicated dilution of number of cells							Pearson's correlation	Significance
		0	**10**	**25**	**50**	**75**	**90**	**100**		
ldcC	10^8	0	1148	696	314	218	178	100	0.449	0.312
	10^7	0	115	120	111	108	105	100	0.998**	0.000
	10^6	0	120	99	103	96	102	100	0.999**	0.000
Spc	10^8	0	611	365	168	132	126	100	0.801*	0.030
	10^7	0	121	128	112	109	107	100	0.997**	0.000
	10^6	0	87	93	93	101	99	100	0.999**	0.000
lys R25	10^8	0	218	163	107	84	85	100	0.969**	0.000
	10^7	0	76	84	89	89	96	100	0.997**	0.000
	10^6	0	92	95	80	102	102	100	0.995**	0.000
lys G25	10^8	0	379	236	119	131	106	100	0.937**	0.002
	10^7	0	89	95	103	104	104	100	0.999**	0.000
	10^6	0	100	82	78	77	85	100	0.985**	0.000
lpp R3	10^8	0	325	215	129	118	120	100	0.963**	0.001
	10^7	0	73	89	97	103	96	100	0.999**	0.000
	10^6	0	78	80	86	90	94	100	0.997**	0.000
lpp R4	10^8	0	101	234	100	93	103	100	0.944**	0.001
	10^7	0	89	104	108	108	106	100	0.998**	0.000
	10^6	0	77	83	86	92	96	100	0.998**	0.000
tat H5	10^8	0	250	190	98	94	98	100	0.970**	0.000
	10^7	0	80	95	97	102	104	100	0.999**	0.000
	10^6	0	83	89	87	96	95	100	0.998**	0.000
tat H9	10^8	0	245	220	134	119	114	100	0.966**	0.000
	10^7	0	94	109	107	106	105	100	0.999**	0.000
	10^6	0	103	100	94	92	93	100	0.998**	0.000
tat H14	10^8	0	489	192	126	132	106	100	0.931**	0.002
	10^7	0	118	125	106	106	103	100	0.998**	0.000
	10^6	0	84	88	92	93	97	100	0.999**	0.000

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

3.4.1.3 Precision

The next step of the validation process was to determine the precision of the bioluminescent method. Mean RLU measurements of ten replicates are depicted in Table 3.7. The percentage Relative Standard Deviation (%RSD) of undiluted cultures (10^8 CFU/mL) and serially diluted cultures for all the strains remained less than 30%. These dilutions for all strains therefore passed the acceptance criteria.

Table 3.7: Relative standard deviation for precision analysis

		10^8	10^7	10^6	10^5	10^4	10^3	10^2
lys R25	**Mean**	6322442	1861133	123430	6517	733	255	255
	SD	70662.5	109515.4	8405.4	162.4	60.3	20.5	10.1
	RSD (%)	1.1	5.9	6.8	2.5	8.2	8.0	4.0
lys G25	**Mean**	2267296	1895567	126476	8821	888	261	192
	SD	102029.5	71422.8	9584.5	246.2	22.7	29.7	30.3
	RSD (%)	4.5	3.8	7.6	2.8	2.6	11.4	15.8
ldcC	**Mean**	45032	59773	6929	716	227	161	151
	SD	581.1	1054.0	135.6	66.5	29.7	38.9	27.2
	RSD (%)	1.3	1.8	2.0	9.3	13.1	24.2	18.1
lpp R3	**Mean**	337625	170840	31230	2615	430	212	150
	SD	30466.0	16758.1	3113.8	49.3	31.5	29.0	18.4
	RSD (%)	9.0	9.8	10.0	1.9	7.3	13.7	12.3
lpp R4	**Mean**	2935995	1928565	255642	22804	2400	351	144
	SD	12330.6	15099.7	21503.8	358.6	103.6	39.7	18.6
	RSD (%)	0.4	0.8	8.4	1.6	4.3	11.3	12.9
spc	**Mean**	199261	150778	30598	2640	455	265	140
	SD	1269.8	4332.2	880.6	76.1	15.9	26.0	27.1
	RSD (%)	0.6	2.9	2.9	2.9	3.5	9.8	19.4
tat H5	**Mean**	2179922	1245812	139368	16680	1796	377	192
	SD	57150.3	25666.8	4830.7	579.9	55.8	31.2	47.2
	RSD (%)	2.6	2.1	3.5	3.5	3.1	8.3	24.6
tat H9	**Mean**	276691	125557	11092	1888	438	161	150
	SD	14690.3	3032.4	184.1	57.1	24.0	28.4	15.5
	RSD (%)	5.3	2.4	1.7	3.0	5.5	17.6	10.3
tat H14	**Mean**	702540	266494	77984	6805	812	231	143
	SD	10189.0	9845.9	1116.6	61.8	27.8	15.0	37.3
	RSD (%)	1.5	3.7	1.4	0.9	3.4	6.5	26.0

Mean RLU measurements, standard deviation (SD) and relative standard deviation (RSD) are indicated at each serial dilution performed in ten replicates.

One-way analysis of variance and F-tests were conducted to determine the significance of variability in the precision data. Using the F-tables, the critical value from an F-distribution with 2, 15 degrees of freedom was 3.68. The F-values (Table 3.8) obtained for all bioluminescent strains were less than the critical value and therefore there was no significant difference between the bioluminescent method and the plate counting method. This was further confirmed by analysis of variance where the significance values were greater than 0.05, implying that there was no significant difference between the two methods.

Table 3.8: F-test and analysis of variance for precision analysis.

Strain	**F-test (P<0.05)**	
	F-value*	Significance
ldcC	1.278	0.307
spc	1.276	0.308
lysR25	1.224	0.322
lysG25	1.250	0.315
lppR3	1.275	0.308
lppR4	1.242	0.317
tatH5	1.255	0.313
tatH9	1.276	0.308
tatH14	1.271	0.309

*Critical value with 2, 15 degrees of freedom at a 0.05 significance level was 3.68.

3.4.1.4 Equivalence

Equivalence of the bioluminescent and plate counting method was determined by performing two-way ANOVA on results obtained from the precision analysis. Table 3.9 shows the statistical significance values for each of the bioluminescent constructs and shows that there was no significant difference (P<0.05) between the bioluminescent value and CFU count for each theoretical CFU value. Hence the bioluminescent method and plate count method are equivalent.

Table 3.9: Equivalence analysis.

Strain	Theoretical value	CFU	RLU	ANOVA (P< 0.05)
lys R25	1.00E+08	1.70E+08	6.32E+06	0.505
	1.00E+07	1.70E+07	1.86E+06	
	1.00E+06	1.70E+06	1.23E+05	
	1.00E+05	1.70E+05	6.52E+03	
	1.00E+04	1.70E+04	7.33E+02	
	1.00E+03	1.70E+03	2.55E+02	
lys G25	1.00E+08	1.70E+08	2.27E+06	0.49
	1.00E+07	1.70E+07	1.90E+06	
	1.00E+06	1.70E+06	1.26E+05	
	1.00E+05	1.70E+05	8.82E+03	
	1.00E+04	1.70E+04	8.88E+02	
	1.00E+03	1.70E+03	2.61E+02	
ldcC	1.00E+08	1.60E+08	4.50E+04	0.486
	1.00E+07	1.60E+07	5.98E+04	
	1.00E+06	1.60E+06	6.93E+03	
	1.00E+05	1.60E+05	7.16E+02	
	1.00E+04	1.60E+04	2.27E+02	
	1.00E+03	1.60E+03	1.61E+02	
lpp R3	1.00E+08	1.51E+08	3.38E+05	0.499
	1.00E+07	1.51E+07	1.71E+05	
	1.00E+06	1.51E+06	3.12E+04	
	1.00E+05	1.51E+05	2.62E+03	
	1.00E+04	1.51E+04	4.30E+02	
	1.00E+03	1.51E+03	2.12E+02	
lpp R4	1.00E+08	1.56E+08	2.94E+06	0.51
	1.00E+07	1.56E+07	1.93E+06	
	1.00E+06	1.56E+06	2.56E+05	
	1.00E+05	1.56E+05	2.28E+04	

	1.00E+04	1.56E+04	2.40E+03	
	1.00E+03	1.56E+03	3.51E+02	
spc	1.00E+08	1.54E+08	1.99E+05	0.494
	1.00E+07	1.54E+07	1.51E+05	
	1.00E+06	1.54E+06	3.06E+04	
	1.00E+05	1.54E+05	2.64E+03	
	1.00E+04	1.54E+04	4.55E+02	
	1.00E+03	1.54E+03	2.65E+02	
tat H5	1.00E+08	1.69E+08	2.18E+06	0.489
	1.00E+07	1.69E+07	1.25E+06	
	1.00E+06	1.69E+06	1.39E+05	
	1.00E+05	1.69E+05	1.67E+04	
	1.00E+04	1.69E+04	1.80E+03	
	1.00E+03	1.69E+03	3.77E+02	
tat H9	1.00E+08	1.58E+08	2.77E+05	0.49
	1.00E+07	1.58E+07	1.26E+05	
	1.00E+06	1.58E+06	1.11E+04	
	1.00E+05	1.58E+05	1.89E+03	
	1.00E+04	1.58E+04	4.38E+02	
	1.00E+03	1.58E+03	1.61E+02	
tat H14	1.00E+08	1.59E+08	7.03E+05	0.491
	1.00E+07	1.59E+07	2.66E+05	
	1.00E+06	1.59E+06	7.80E+04	
	1.00E+05	1.59E+05	6.81E+03	
	1.00E+04	1.59E+04	8.12E+02	
	1.00E+03	1.59E+03	2.31E+02	

RLU measurements and CFU count for all bioluminescent constructs at each serial dilution. Statistical significance is shown at $P<0.05$.

3.4.2 Quantitative Polymerase Chain Reaction (qPCR)

To determine plasmid copy numbers and to ascertain if the plasmid remained stable during the course of the study, qPCR experiments were performed. Several PCR reactions were performed to optimise the annealing temperatures for successful amplification of both dxS and gentamicin genes. Finally, the annealing temperatures were set at 46^{o}C and 60^{o}C for dxS and gentamicin amplification, respectively. Quantitative PCR amplification products of gentamicin (Figure 3.6 A) and DxS (Figure 3.6 B) for five bioluminescent strains spc, ldcC, lppR4, lysR25 and tatH5 were further resolved by gel electrophoresis for visual satisfaction.

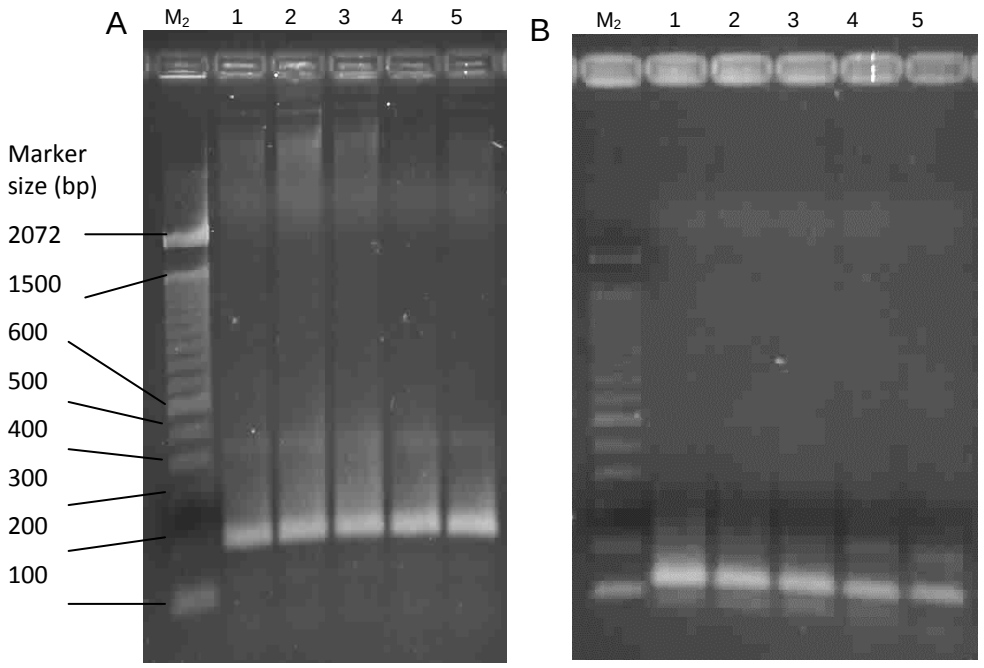


Figure 3.6: Quantitative PCR reactions for gentamicin and dxS amplification. Reactions were performed using final optimised conditions for amplification of gentamicin gene (A) and dxS gene (B) for the strains of spc, ldcC, lppR4, lysR25 and tatH5 in lanes 1, 2, 3, 4 and 5, respectively, in both cases.

Standard calibration curves were constructed for genomic and plasmid DNA (Figure 3.7). A range of concentrations of genomic and plasmid DNA were used to perform quantitative PCR to determine their C_t values. As the concentration of plasmid (Figure 3.7A) and genomic DNA (Figure 3.7C) increased, the C_t value decreased. Melt curve analysis for both plasmid amplification (Figure 3.7B) and genomic amplification (Figure 3.7D) also indicated single maxima at approximately 85^{o}C, suggesting that pure PCR products were obtained.

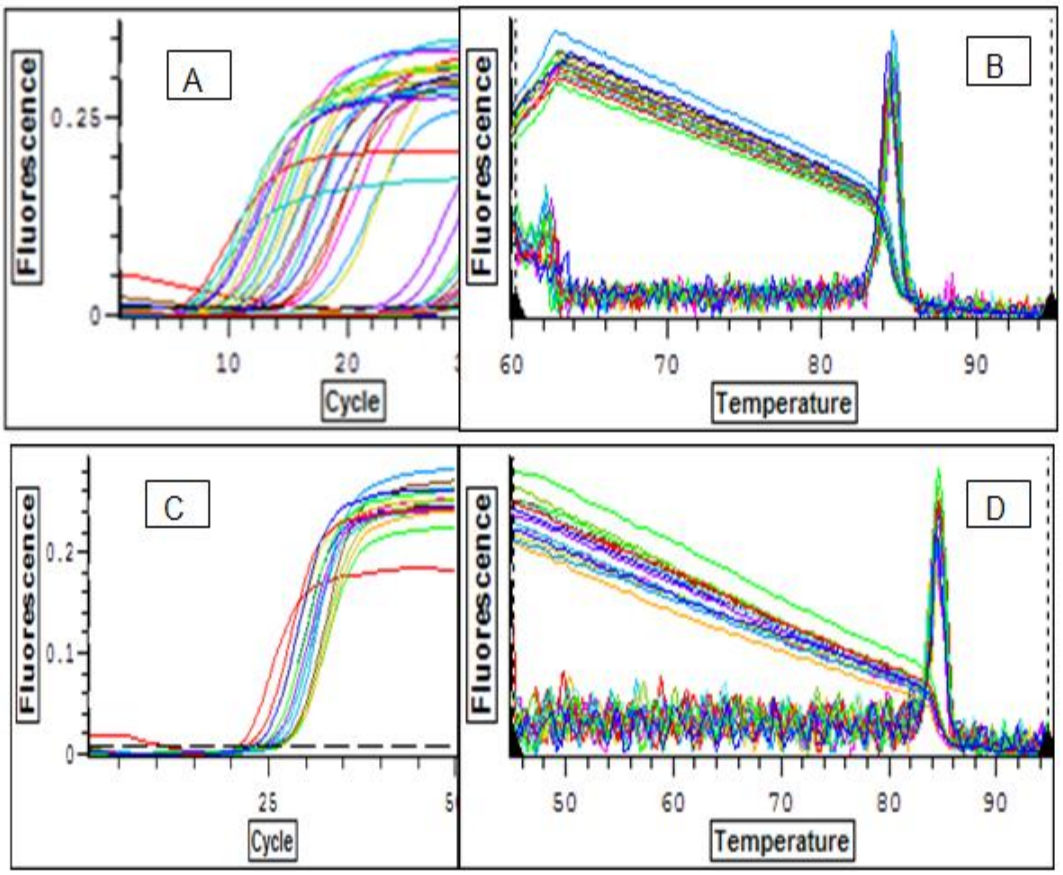


Figure 3.7: Standard calibration and melt curves for plasmid and genomic DNA standards. Figure A and B correspond to calibration curves and melt curves, respectively, for plasmid DNA, while figure C and D refer to calibration curves and melt curves, respectively, for genomic DNA. Serial dilutions of plasmid and genomic DNA extracted from *Pseudomonas* bioluminescent constructs were used for quantification of genomic and plasmid DNA by real-time PCR for the construction of calibration curves.

Calibration curves for genomic and plasmid DNA, were generated (Figure 3.8). C_t values were found to decrease proportionately as the concentration of DNA template increased in each case. The slope for both curves was computed to be -3.40 for genomic DNA and -3.57 for plasmid DNA. The R^2 values were found to be greater than 0.98 indicating high efficiency of PCR. The efficiencies of the standard curves were derived to be 96.8% for genomic and 90.7% for plasmid DNA.

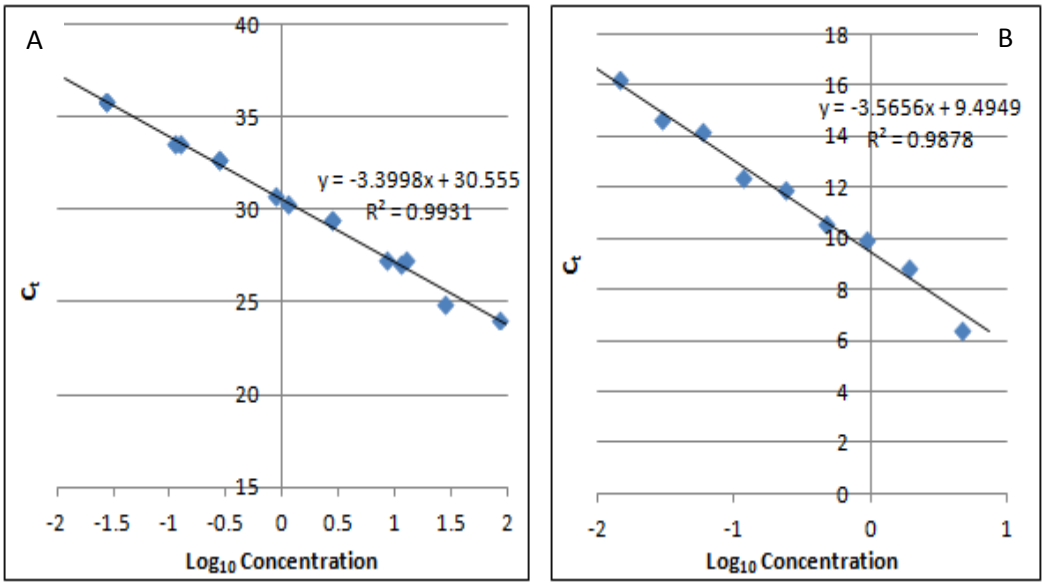


Figure 3.8: Calibration curves for genomic (A) and plasmid (B) DNA amplification. Quantitative PCR was performed using various concentrations of template DNA. C_t values are plotted against DNA concentration on a $\log_{10}$ scale. Linear inverse relationship between C_t values and $\log_{10}$ DNA concentration is observed and the corresponding correlation coefficients are shown.

Samples of total DNA extracted from bacterial cultures of all strains at defined time points along the growth curve were analysed by qPCR. Examples of C_t values and melt curve analysis for genomic and plasmid amplification are shown in Figure 3.9. As expected, the wild-type strain lacked plasmid DNA and therefore Figure 3.9C indicated an amplification plot with a C_t value of 31, which was significantly different to C_t values obtained for all other strains with plasmids.

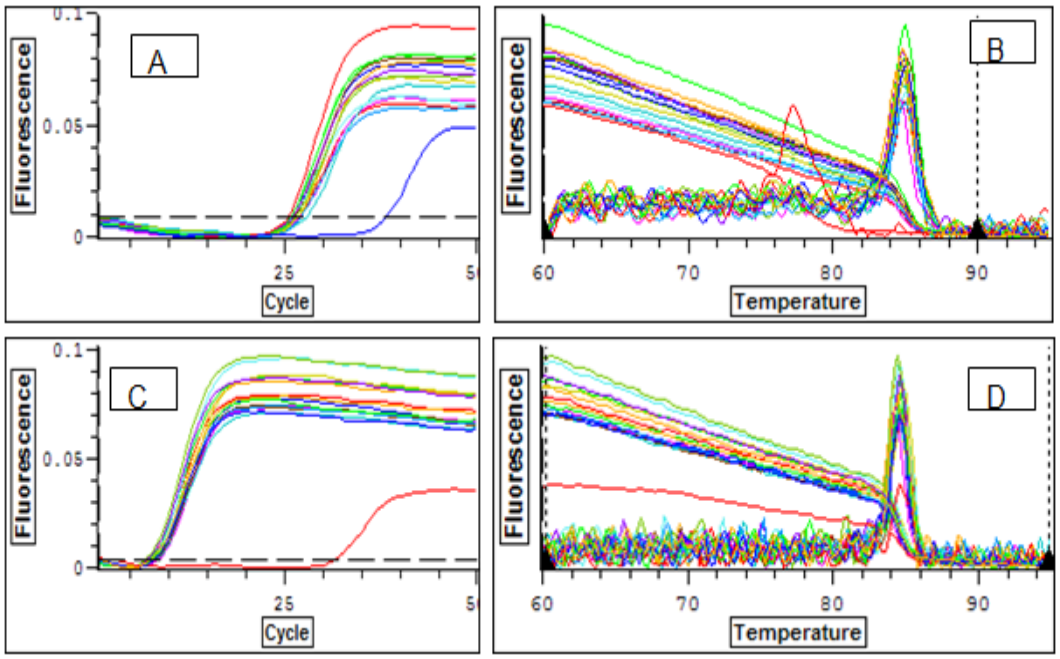


Figure 3.9: Amplification plots and melt curves for genomic and plasmid DNA samples. A and B corresponds to amplification plots and melt curves, respectively, for genomic DNA extracted from 24 hour cultures. C and D refer to amplification plot and melt curves, respectively, for plasmid DNA extracted from 24 hour cultures. Total DNA extracted from *Pseudomonas* bioluminescent constructs at various time points along their growth kinetics were used for quantification of genomic and plasmid DNA by real-time PCR.

C_t values were obtained and plasmid copy numbers for each strain calculated at pre-defined time points (Appendix 8, Table 7.3). By plotting the plasmid copy number profiles for all plasmid-bearing strains (Figure 3.10), it was shown that plasmid copy number for all plasmids was greatest at 6 hours, with 100 and 120 copies for constructed variants and pME4510 plasmids, respectively. Plasmid copy number then decreased greatly by 24 hours with recombinant variants showing approximately 50 copies and pME4510 showing approximately 80 copies. The PCN after 24 hours remained consistent throughout the 28 days. Correlations between time and plasmid copy number were weak and insignificant ($P<0.05$) (Appendix 8, Table 7.4). Weak relationships between plasmid copy number and time suggested that plasmids remained stable throughout the duration of the study.

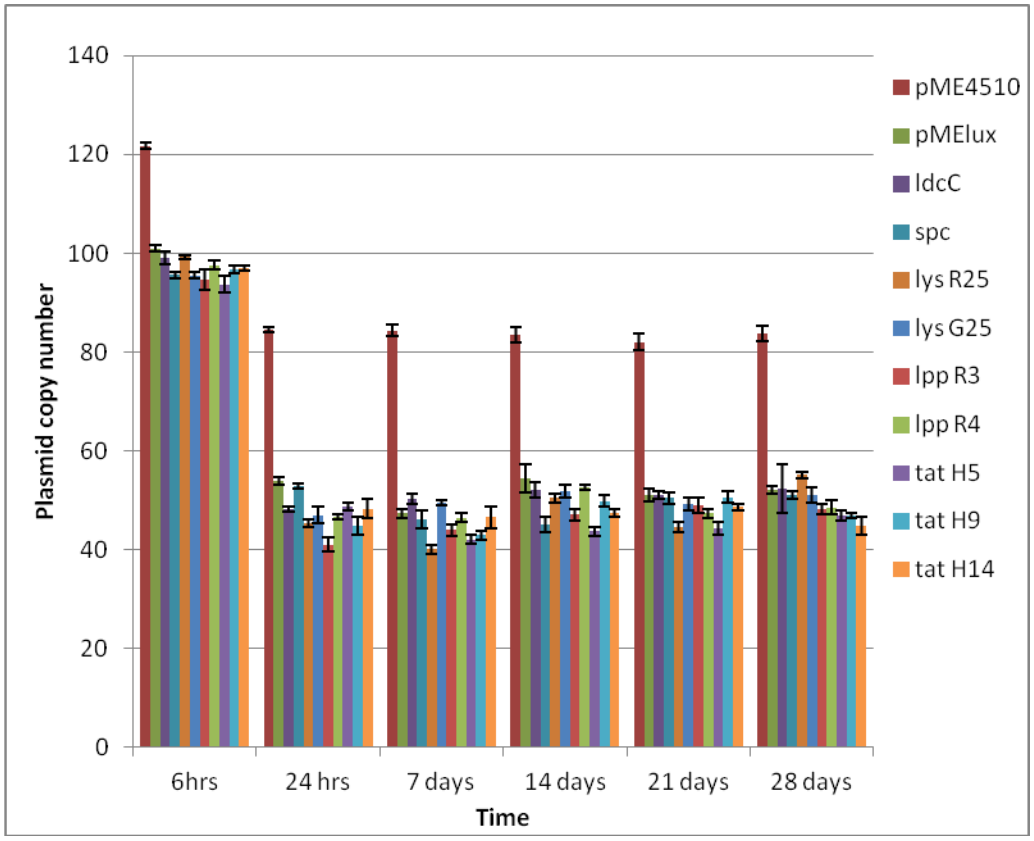


Figure 3.10: Plasmid copy number analysis for all plasmid-bearing strains. Plasmids copy numbers are represented at exponential phase (6 hours) and stationary phase (from 24 hours up to 28 days). Error bars represent standard deviations from n=3.

3.5 Discussion

Novel rapid methods of microbial detection need to be validated and compared with existing traditional methods that are approved by various bodies. The validation parameters for the bioluminescent method were therefore addressed according to various documents (Kramer et al., 2008; PDA, 2000). The parameters validated were linearity, accuracy, precision, equivalence and limit of detection and quantification.

3.5.1 Linear range

The bioluminescence and CFU counts for all cultures of *Pseudomonas* bioluminescent constructs were measured at a series of dilutions after 24 hours of growth. Bioluminescence readings were found to reduce at a linear rate with increase in dilution factor (Figure 3.4), meaning that as the number of cells decreased the production of bioluminescence reduced at an equal rate. The range where the rate of increase was linear was between 10^4 and 10^8 CFU/mL for strong promoters and 10^5 to 10^8 CFU/mL for weak promoters. Correlations between RLU and CFU were found to be strong ($r > 0.97$) and significant ($P < 0.01$) within the range 10^3 to 10^7 cells. Correlations coefficients decreased slightly when undiluted cultures were incorporated into the range. However, these correlations were also found to be significant. Good correlations were also found by another study (Neilson, Pierce & Maier, 1999) which showed that there was a linear relationship between bioluminescence and cell number. Furthermore, Neilson and his colleagues also showed that the amount and timing of bioluminescence is highly dependent on changes in cell numbers.

It was observed that the ratio of bioluminescence to CFU count for undiluted cultures were less than those of 10-fold diluted cultures, implying that undiluted cultures hindered the accurate detection of bioluminescence. This was because the high number of bacterial cells in

undiluted cultures caused cultures to be turbid, thereby diffracting light. This is called the inner filter effect (Kao, Asanov & Oldham, 1998). The other possiblity of detector saturation, whereby bioluminescence is measured above the upper limit of detection, was omitted since the bioluminescence produced was less than the saturation threshold, which is $1x10^8$ RLU as specified by the manufacturer. Additionally the bioluminescence produced by weak promoters resulted in a similar turbidity effect compared to that of strong promoters even though the RLU from the undiluted cultures of weak promoters were less than that of 10-100 fold of diluted cultures with strong promoters. Furthermore, the linearity validation indicated that the requirement of 10^6 cells inocula for preservative efficacy testing was within the linear range and therefore the inner-filter effect would not affect the preservative testing regimes.

Consistent ratios of log RLU per log CFU were observed for 10-fold and 100-fold diluted samples, indicating that RLU reading reduced proportionally as CFU count also reduced. The relationship between RLU and CFU weakened beyond 10^4 fold dilution for weak promoters and beyond 10^5 fold dilutions for strong promoters since bioluminescence of such dilute cultures was similar to the background value.

It was observed that both strains of lys and lpp produced similar RLU readings per CFU. Strains harbouring ldcC and spc gave the lowest light output. Strain tat S5 produced three-fold and two-fold greater bioluminescence than its variants tat S9 and tat S14, respectively. The variation in bioluminescence per CFU for different variants of the same bioluminescent strain may be explained by the fact that there is a variation in plasmid copy number per cell or the gene expression is dependent on plasmid topology and conformation. The fact that DNA conformation and topology is a factor for strength of gene expression has been was noted previously in numerous studies (Mishra et al., 1990; Palecek, 1991; Travers, 1987; Werel et al., 1991). Furthermore, plasmid topology is a factor of plasmid DNA sequence. However, after full plasmid sequencing analysis, no significant differences in plasmid sequences between the

three variants of tat were found. Hence, the plasmid copy numbers for the strains were investigated.

3.5.2 Limit of quantification, detection and sensitivity

The lower limit of detection was estimated to be 10^2 RLU as observed by the negative control strains with respect to bioluminescence. This showed that further serial dilutions produced bioluminescence close to the lower limit of detection of the luminometer and therefore the RLU:CFU ratios did not provide a true reflection of the health state of the bacterial cells. Sensitivity of the bioluminescent method was determined to be 10^3 CFU/mL and therefore the bioluminescent method has proved to be highly sensitive. The lower limit of quantification was 10^3 CFU/mL, which is also reported in the validation document (PDA, 2000) and with studies using ATP bioluminescence (Kramer et al., 2008). According to a review of methods for rapid microbiological assay (Shintani, Sakudo & McDonnel, 2011), the sensitivity of the ATP bioluminescent method was quoted as greater than 10^2-10^3 CFU/mL. However, a disadvantage of the ATP bioluminescent method was that there is a high rate of false positive results due to the fact that non-microbial ATP cannot be distinguished from microbial ATP and in particular microbial ATP from luciferase pathway (Kramer et al., 2008). Furthermore, the ATP bioluminescent method requires cell lysis and extraction of ATP for each sample. This can be very labourious and time-consuming, requiring destructive sampling, and therefore does not provide data in a ‘real-time’ mode.

3.5.3 Accuracy studies

Accuracy of the bioluminescent method was assessed by determining the percentage recovery of undiluted cultures and serially diluted cultures. Accuracy assessment of the bioluminescent method does not determine the absolute number of bacterial cells in a sample and therefore 'percentage recovery' of cells was utilised to determine how accurate the bioluminescent method was compared to the traditional plate counting method. For samples to pass the accuracy validation, the percentage recovery was required to be between 70% and 130% (PDA, 2000).

Percentage recoveries for undiluted cultures were outside the acceptance criteria. Undiluted samples indicated wider fluctuations in bioluminescence measurements, thereby affecting the recovery process. These fluctuations were probably not due to fluctuations in gene expression but rather due to inner filter effects caused by highly turbid cultures (Kao et al., 1998). This trend has been reported previously (Lei, Mulchandani, Chen, Wang & Mulchandani, 2003; Mulchandani, Chen & Mulchandani, 2006; Mulchandani, Lei, Chen, Wang & Mulchandani, 2002). However, percentage recoveries of 10-fold and 100-fold diluted cultures were found to be within the acceptance criteria and therefore passed the accuracy validation analysis. Furthermore, correlations between RLU and CFU were found to be significant ($P<0.01$) within the diluted suspensions of each serial dilution tested. Hence the bioluminescent method was very accurate within the linear working range and thus the bioluminescent method as a microbial testing method passed the criteria set out in accordance with various documents (ISO, 1994a, 1994b, 1994c; PDA, 2000).

3.5.4 Precision and equivalence

Precision measures the repeatability of any method by measuring the relative standard deviation within replicate samples. The acceptance criteria require that the relative standard deviation is less than 30% of the variation within replicates of the samples to pass the precision assessment (PDA, 2000). Furthermore, one-way analysis of variance and an F-test determined the significance of variability in the precision data. The F-test showed that there was no significant difference between the bioluminescent method and the plate counting method for all bioluminescent strains, since the F-values were less than the critical value (3.68). This was further confirmed by analysis of variance, where the significance values were greater than 0.05, implying that there was no significant difference between the two methods. Therefore, the bioluminescent method as a microbial testing method passed the criteria set out in accordance with various documents (ISO, 1994a, 1994b, 1994c; PDA, 2000). The % RSDs for all dilutions for all the strains tested were found to be less than 30%. The maximum of % RSDs for 10^2 CFU/mL was 26%, which was still less than 30%. This was because bioluminescence at 10^2 CFU/mL is undetectable due to the lower limit of detection. According to Kramer *et al.* (2008), the precision of the ATP bioluminescence method was found to be 24.9% and 26% for 10^8 CFU/mL and 10^4 CFU/mL, respectively. The precision of the bioluminescent method using the bioluminescent constructs designed in this study was less than 10% and less than 15% for 10^8 CFU/mL and 10^4 CFU/mL, respectively. This indicated that the whole cell microbial bioluminescent method may be more precise than the ATP bioluminescent method, even though the ATP bioluminescent method is accepted as an alternative method for testing preservatives according to the pharmacopoeias.

Equivalence measures the variability between the traditional plate counting method and the bioluminescent method. Two-way ANOVA showed no significant difference between the two methods for all bioluminescent strains. No significant difference between the

bioluminescent and the plate counting methods was also shown by other studies (Quilliam, Williams & Jones, 2012; Sun, Connor, Pennington & Lawrenz, 2012; Thorn, Nelson & Greenman, 2007). A study comparison between CFU count and ATP bioluminescence also showed that both methods were equivalent (Kramer et al., 2008).

3.5.5 Plasmid copy number

Plasmid copy numbers for all strains were evaluated with the objective of determining plasmid stability throughout 28 days as well as the underlying reasons for differences in gene expression by different variants of bioluminescent constructs containing tat promotors. Plasmid copy numbers were determined by adapting the qPCR methods used previously (Lee et al., 2006b). Establishing the rate of amplification of the dxS and gentamicin genes on the genome and plasmid, respectively, provided the basis for comparing the numbers of plasmid copies per genomic DNA. This was achieved by determining the relationship between DNA concentration and C_t value. It was found that C_t values decreased as DNA concentration increased as expected; therefore, there was an inverse relationship between C_t value and DNA concentration. Correlation coefficients of 0.993 and 0.988 and negative slopes for genomic and plasmid DNA, respectively, also indicated a strong inverse relationship between DNA concentration and C_t values for both plasmid and genomic DNA.

C_t values can be affected by the efficiency of PCR reactions. Theoretically, amplification efficiency is regarded as 1 when fluorescence doubles with every cycle. Practically, this may not be the case. Hence efficiencies between 90-110%, calculated from the slope of a calibration curve being between -3.3 and -3.6 are acceptable (Applied Biosystems; Fraga et al., 2008; Wong & Medrano, 2005). Calibration curves for genomic and plasmid DNA were generated with high efficiencies of -3.40

and -3.57 for genomic and plasmid DNA, respectively, as exemplified from the slopes. This equated to efficiencies of 96.8% and 90.7% for genomic and plasmid DNA, respectively.

Melt curve analysis was performed after amplification of plasmid and genomic DNA segments. Single peaks were obtained for amplification products of both DxS and gentamicin genes, indicating that there was no non-specific amplification and that the products of amplification were pure.

Plasmid copy numbers of all plasmid-bearing strains were determined at various time points along the growth and death curves, up to 28 days. This was achieved by determining the concentrations of plasmid DNA and genomic DNA at various time points and computing them to determine plasmid copy number per genome. As shown in Figure 3.10, plasmid copy number for all strains was greatest during the exponential phase with pME4510 strain showing 120 copies and the recombinant constructs showing 100 copies. This is the phase when the cells are dividing exponentially and therefore the plasmids are replicating before the cells replicate. During stationary phase, plasmid copy numbers were approximately 80 and 50 for strain pME4510 and recombinant constructs, respectively. This means that increase in plasmid size from 4kb for pME4510 to 10kb for the recombinant constructs was associated with reduced plasmid copy number. Previous studies have also shown that plasmid copy number decreases as plamid size increases (Smith & Bidochka, 1998). This is because greater amounts of resources are required to maintain and replicate larger plasmids compared to those required for smaller ones. All plasmid copy numbers remained consistent throughout the 28 days, indicating that the plasmids were stably maintained by the cells. This was further confirmed by a lack of correlation between time and plasmid copy number that were also insignificant ($P<0.05$) for each of the strains tested. Stability of plasmids was regarded as vital for this study since preservative efficacy tests required stable plasmids to be maintained throughout the duration of the test. Furthermore, instability of certain plasmids as observed in chapter 2 led to

the investigation of plasmid stability in these working bioluminescent strains. Also, the variation in bioluminescence between variants containing the same promoter sequence was not due to differences in plasmid copy number.

3.6. Conclusion

Validation of the bioluminescent constructs was successful. The linear range was between 10^4 to 10^8 CFU/mL for strong promoters and between 10^5 to 10^8 CFU/mL for weak promoters and bioluminescence increased proportionately with increase in number of cells. The lower limit of detection was estimated to be 10^2 RLU as observed by the negative control strains with respect to bioluminescence. The lower limit of quantification was 10^3 CFU/mL. Accuracy of the bioluminescent method was assessed by determining the percentage recovery of cultures; 10-fold and 100-fold diluted cultures were found to lie within the acceptance criteria and therefore passed the accuracy validation analysis. Precision measures the repeatability of any method by measuring the relative standard deviation within replicate samples. The % RSDs for all dilutions for all the strains tested were found to be less than 30% and hence the samples passed the precision assessment. Plasmids were stably maintained by the cells throughout 28 days.

This study has therefore shown that the bioluminescent method passed all the criteria required for validation. All the constructs have therefore been successfully validated and the bioluminescent method shown to be at least equivalent if not better than the traditional plate counting method and the ATP determination method. The newly created recombinant constructs are functional and stable and therefore may be utilised for further studies.

Chapter 4: Bacterial growth and gene expression

4.1 Introduction

4.1.1 Growth and death kinetics

Many natural environments present unfavourable conditions for bacterial growth and division, such as low nutrient availability, less favourable temperature, pH and osmolarity. However, bacteria have evolved strategies to survive under such unfavourable conditions and subsequently grow under favourable conditions. This phenomenon of growth and survival is termed a 'feast or famine' phenomenon. Nevertheless, most studies and experiments involving bacteria are performed in closed systems. In systems such as the laboratory bacterial batch cultures where conditions are optimum, bacterial cells go through five phases of growth in sequence (Figure 4.1).

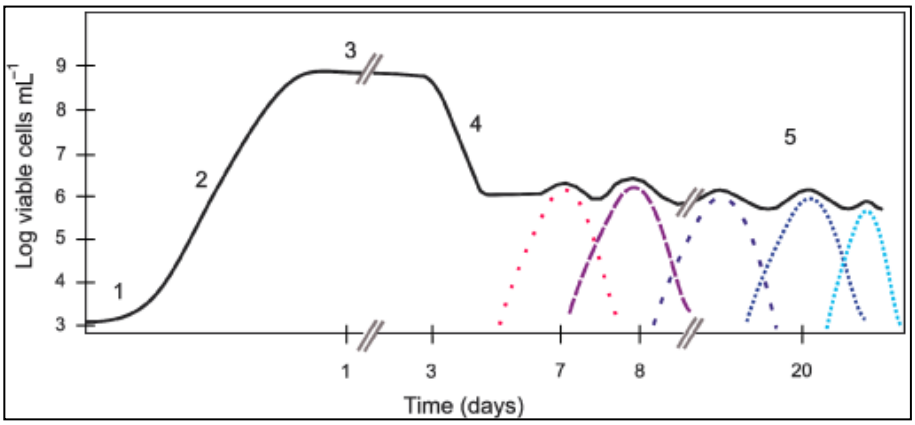


Figure 4.1: Bacterial growth phases. Cells growing in a closed system under optimum conditions. Numbers 1-5 represent lag, exponential, stationary, death and long-term stationary phases, respectively. Dotted coloured lines represent the GASP phenotype (Finkel, 2006; Navarro Llorens, Tormo & Martinez-Garcia, 2010).

The lag phase is characterised by growth in cell volume but not in numbers, increased metabolism and synthesis of proteins, RNA and DNA (Al-Qadiri et al., 2008). Considering that the source of cells at inoculation arrives from the stationary phase of previous batches, cells become rapidly adapted in their new environment during early lag phase; this represents the transition from stationary phase (Rolfe et al., 2012). During

this phase there is a high concentration of RNA polymerase (Klumpp & Hwa, 2008; Proshkin, Rahmouni, Mironov & Nudler, 2010). Transcription for proteins required for lag and log phases of growth is initiated because certain promoters possess a strong binding affinity for RNA polymerase (Rolfe et al., 2012). It is thought that collection of these proteins beyond a certain threshold may be an indicator for cells to enter the exponential phase of growth (Rolfe et al., 2012).

The exponential or log phase of growth occurs where the environment is conducive to the growth of cells and therefore the cells are actively dividing at a constant rate (Al-Qadiri et al., 2008; Navarro Llorens et al., 2010). The rate of growth at this stage is considered in terms of the doubling time or generation time (Al-Qadiri et al., 2008). Although the growth rate is constant, it does depend on various factors such as media type, temperature, space and oxygen availability.

The stationary phase features a balance between cell division and cell death. During this phase, populations consist of a mixture of healthy cells and damaged, dying cells. Nutrients become limiting, metabolites increase in concentration and there is spatial congestion due to the increased number of cells. Certain bacteria also produce secondary metabolites during this phase, while other bacteria prepare for sporulation (Al-Qadiri et al., 2008). However, when bacteria from the stationary phase are inoculated into a fresh medium, the degenerative process stops and instead the bacteria prepare for growth and division, meaning the cells enter the lag phase. This process is termed reversible conditional senescence (Nystroem, 2007). Hence, bacterial cells in their lag phase rejuvenate, repair oxidative damage and prepare for growth and division.

The death phase is symbolized by an exponential decrease in number viable cells due to reduction in cellular energy, changes in pH and further accumulation of metabolites (Al-Qadiri et al., 2008). Nutrients are then released back to the medium, since 99% of the cells die and degrade. However, the surviving cells exploit this new environment to their

advantage and utilise the nutrients released for their growth. This leads to the long-term stationary phase (Finkel, 2006), where there are repeated rounds of cell death and re-growth of survivors; each time, the surviving population shows a difference in phenotype. For example, the surviving cells are better adapted to utilize nutrients and grow in a metabolite-rich medium. This phase is characterised by several phenotypes: the growth advantage in stationary phase phenotype, viable but non-culturable states as well as a stationary phase contact dependent inhibition state (Navarro Llorens et al., 2010).

The GASP phenotype is defined as the ability of mature bacterial cells from long-term cultures to outcompete young cells from fresh cultures (Zambrano & Kolter, 1996; Zinser & Kolter, 2004). Mature cells have an advantage over fresh young cells due to their ability to scavenge glucose, ammonia and amino acids (Navarro Llorens et al., 2010).

Certain bacterial cells enter a dormant state upon prolonged starvation. Such cells are viable and metabolically active, albeit with decreased levels of metabolic activity; however, they cannot grow and divide in culture media. Hence, these cells are termed as viable but non-culturable cells (VBNC) (Navarro Llorens et al., 2010; Oliver, 2005). Factors contributing to the VBNC state include starvation, low oxygen concentration and low incubation temperature, amongst others (Rowan, 2010). Cells in the VBNC state have been shown to be resuscitated by changes in certain conditions (Zhong, Chen, Zhang & Jiang, 2009). For instance, a simple temperature shift can resuscitate certain cells. Resuscitated cells are able to express pathogenicity; this is a major problem in the hospital, food and pharmaceutical industries and preservative systems, since it presents a significant risk to human health (Oliver, 2005). These VBNC cells may also initiate new infectious diseases but the risk to human health is even more pronounced because traditional methods of detection will not detect these cells.

Stationary phase contact dependent inhibition (SCDI) (Lemonnier et al., 2008) is a phenomenon where cells undergo mutations as a result of serial culturing and the emerging mutant cells inhibit the growth of parent un-mutated cells. Mutated strains have been shown to produce glycogen in increased amounts.

4.1.2 Bacterial growth rate-dependent gene expression

Bacterial cells replicate rapidly under favourable conditions and such rapid growth and division is tightly integrated and controlled by various metabolic, genetic and biochemical pathways. These global networks synchronize gene expression with various stages of replication in the growth cycle (Scott & Hwa, 2011) in response to changing environment. Quantitative study of gene expression is therefore dependent on the physiological status of the cell. Any change in the state of the cell due to change in the environment is thereby brought about by changes in gene expression. Therefore, growth rate is a factor of constitutive gene expression. Alterations in growth environment dictate changes in cell physiology as characterised by variations in nucleoid structure, density of DNA superhelices and RNA polymerase specificities (Bordes et al., 2003; Sobetzko, Travers & Muskhelishvili, 2012). The amounts of protein and chromatin also change in response to changes in growth rate.

Growth rate-dependent constitutive gene expression can be studied by measuring growth rate-dependent parameters in the cell, such as gene or plasmid copy number or RNA polymerase concentration (Gerosa, Kochanowski, Heinemann & Sauer, 2013; Klumpp & Hwa, 2008). For the purpose of this study, the global gene expression as a factor of growth rate is quantified by measuring constitutive expression of the *lux* cassette placed under constitutive promoters.

4.2 Aim and objectives

The aim of this work was to determine the growth and death pattern of the recombinant constructs as compared to wild-type *P. aeruginosa* ATCC9027.

The objectives of this work were:

- To determine the growth and death patterns of all strains at various time points up to 28 days by constitutive gene expression using the bioluminescence method and traditional plate counting method.
- To compare the data obtained by both methods.
- To evaluate the promoter strengths of bioluminescent constructs by constitutive gene expression at various stages of growth and relate the strengths to promoter sequences.

4.3 Materials and methods

4.3.1 Growth and death kinetics of *P. aeruginosa* wild-type and bioluminescent constructs

The bacterial strains used for growth and death kinetic studies were:

Wild-type *P. aeruginosa* ATCC 9027 (WT)

P. aeruginosa ATCC 9027 + Promoterless pME-*lux*

P. aeruginosa ATCC 9027 + ldcC pME-*lux*

P. aeruginosa ATCC 9027 + spc pME-*lux*

P. aeruginosa ATCC 9027 + lys R25 pME-*lux*

P. aeruginosa ATCC 9027 + lpp R4 pME-*lux*

P. aeruginosa ATCC 9027 + tat H5 pME-*lux*

All these strains were grown as specified in section 3.3.1. Isolated colonies were then inoculated into 100 mL LB containing 10μg/mL gentamicin (except for WT that was inoculated into LB without antibiotic) and incubated at 32^{o}C and 150 rpm shaking conditions for 28 days. Samples were retrieved at each hour upon inoculation from each of the flasks for the first 48 hours followed by sample retrieval at 7 days, 14 days, 21 days and 28 days. All samples were serially diluted 10 fold seven times in buffered peptone water (Oxoid Ltd). Aliquots (1 mL) of each undiluted culture and diluted culture were transferred into culture tubes aseptically for bio-luminescence measurements. Relative light units (RLU) were measured as described in section 2.3.15. Aliquots (100 μL) of each undiluted and diluted culture were transferred onto LB agar plates as described in section 3.3.2.

4.3.2 Growth and death kinetics of *P. aeruginosa* wild-type and recombinant constructs using fluorescence spectroscopy

Strains were grown as described in section 3.3.1. Upon inoculation, samples were retrieved, in triplicate, from each of the flasks after periods of 0 hours, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, followed by sample retrieval at 7 days, 14 days, 21 days and 28 days. Cultures were centrifuged at 13000rpm for five minutes and the supernatants were discarded. Samples were prepared according to manufacturer's instructions (LIVE/DEAD® *Bac*Light Bacterial Viability Kit from Molecular Probes®).

Following staining, 200 μL of each sample was transferred to a black 96-well microtitre plate for measuring fluorescence. The plate was placed in a fluorescent plate reader (Promega Glomax Multidetection system using fluorescent module and fluorescent optical kit) and fluorescence was measured using excitation wavelengths of 490nm and 525nm and emission wavelengths of 510-570nm and 580-640nm for live and dead cells, respectively.

4.3.3 Data analysis

Relative bioluminescences per unit CFU were calculated by determining the ratios of RLU:CFU.

Pearson's correlations between relative bioluminescence, plate count and relative fluorescence reading were performed at a significance level of $P<0.05$.

Two-way analysis of variance (ANOVA) at a significance level of $P<0.05$ was performed between relative bioluminescence, plate count and relative fluorescence data to determine the equivalence between these methods.

4.4 Results

4.4.1 Growth and death kinetics of *P. aeruginosa* wild-type and recombinant constructs

The growth and death kinetics of *Pseudomonas aeruginosa* wild-type and recombinant constructs were evaluated for 28 days. The bioluminescence intensity was measured and compared against colony forming units at each hour up to 48 hours then by every 7 days up to 28 days (Figure 4.2). Bioluminescence intensity was shown to increase proportionally up to 12 hours, followed by stabilisation up to 24 hours. There was a slight decline from 24 hours to 48 hours. Colony forming units (CFU) for all strains were 10^4 for the first 3 hours, indicating a constant lag phase. CFU counts for all bioluminescent strains increased up to 12 hours, reaching 10^8 CFU/mL whilst the CFU counts for the wild-type increased to10^8 CFU/mL by 8 hours. Further growth was observed for the wild-type strain, reaching 10^9 CFU/mL up to 24 hours. However, the growth was at a much reduced rate. A similar pattern to that of the wild-type was observed for the promoterless strain. Stationary phase onset was at 12 hours for all the strains and remained constant up to 48 hours.

Over 28 days, bioluminescence of all five bioluminescent constructs decreased. However, there was an increase in bioluminescence for all five bioluminescent constructs at 14 days. After 14 days, the bioluminescence continued to decrease. After 48 hours, CFU count declined constantly throughout the duration of the experiments, reaching 10^3 CFU/mL. However, wild-type and promoterless CFU count remained stable from 14 and 21 days onwards, decreasing to less than 10^7 and less than 10^5 CFU/mL, respectively.

Overall, the promoter with the greatest bioluminescence during the exponential phase was lpp-pME*lux*, followed by lys-pME*lux*, tat-pME*lux*, ldcC-pME*lux* and spc-pME*lux*. However, during stationary phase, tat-pME*lux* had the greatest bioluminescence, followed by lys-pME*lux* and

lpp-pME*lux* producing similar bioluminescence, and finally ldcC-pME*lux* and spc-pME*lux*.

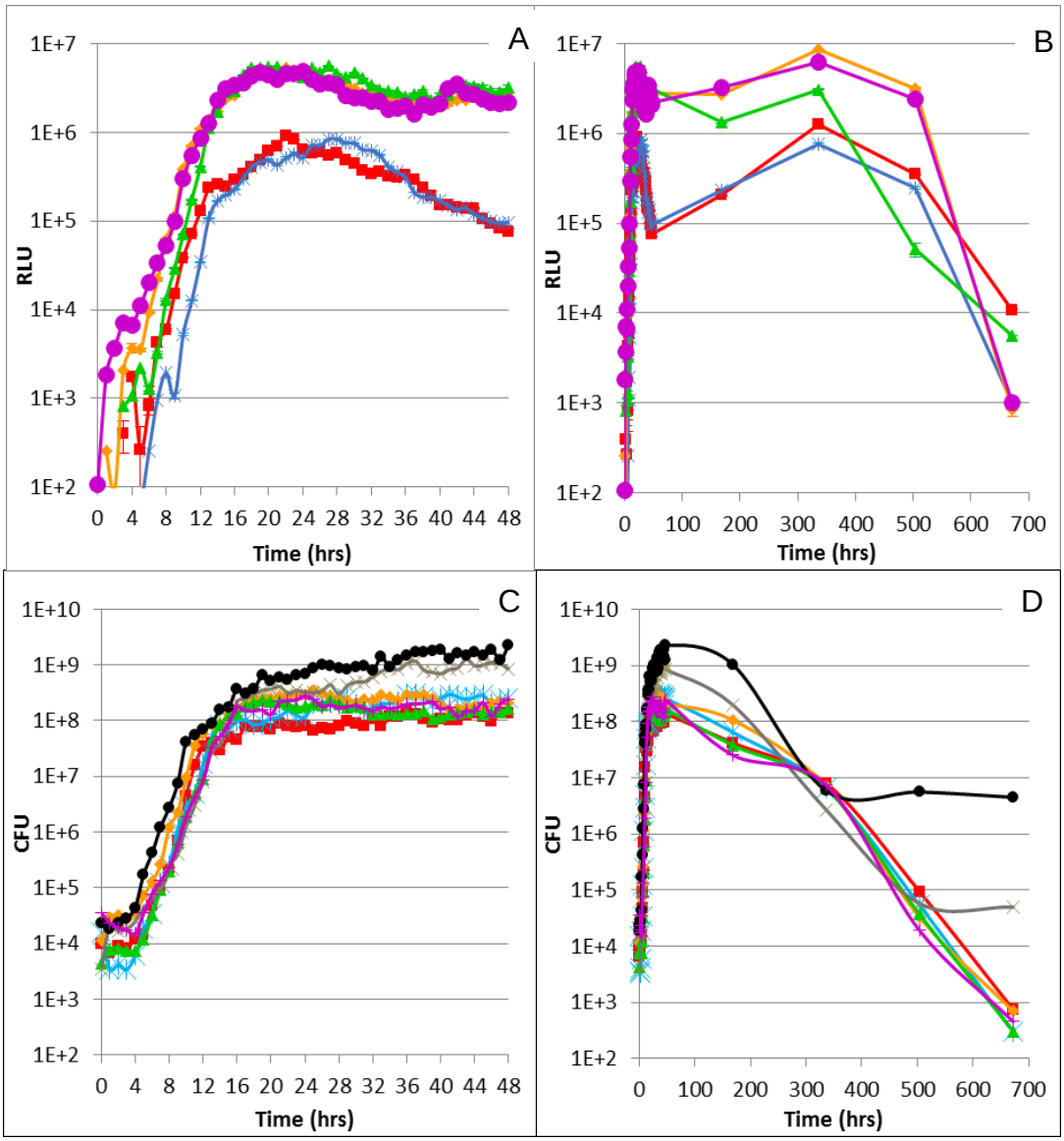


Figure 4.2: Growth curve for wild-type and six constructs. Bioluminescence measurements up to 48 hours and 28 days are shown in figure A and B respectively. CFU determinations up to 48 hours and 28 days are shown in figure C and D respectively. ldcC, spc, lys, tat lpp, pMElux, PA9027 (WT).

To determine the strength of each of the promoters during the growth and death cycle, the ratios of RLU to CFU were assessed (Figure 4.3). During the exponential growth phase, lpp-pME*lux* produced the highest bioluminescence per CFU, peaking at 5 hours with a ratio of 0.45 (Figure 4.3A). This was followed by tat-pME*lux*, ldcC-pME*lux* and lys-pME*lux*, each reaching a maximum at 4 hours, with ratios of 0.18, 0.15 and 0.10, respectively. Finally, spc-pME*lux* reached a maximum at 7 hours with a ratio of 0.001. Constant bioluminescence per CFU was observed for all bioluminescent strains from 12 to 48 hours.

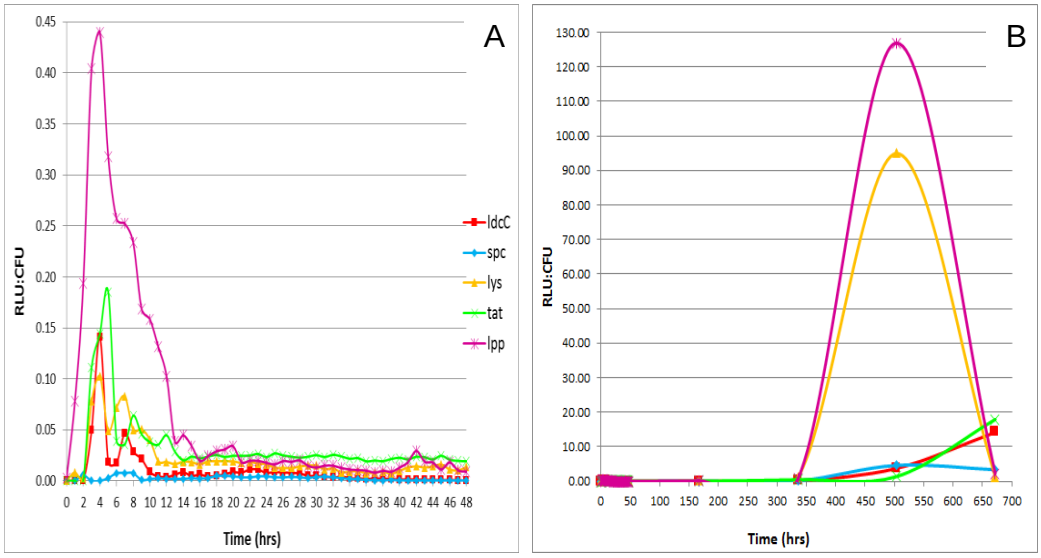


Figure 4.3: Ratio of RLU:CFU for five constructs. A: Up to 48 hours, B: Up to 28 days. Ratios of RLU:CFU are indicative of promoter strength. dcC, pc, s, tat, pp.

After 14 days (336 hours), the ratios did not increase proportionately with increase in time throughout the duration of the assays (Figure 4.3B).

4.4.2 Promoter strength and sequence analysis

The strength of five promoters was evaluated by determining the relative bioluminescence per CFU count, in an attempt to relate each of the strengths to their respective promoter sequences. As illustrated in Figure 4.4, the promoter strength for the five bioluminescent constructs was found to be lpp>tat>lys>ldcC>spc in order of decreasing strength during exponential phase.

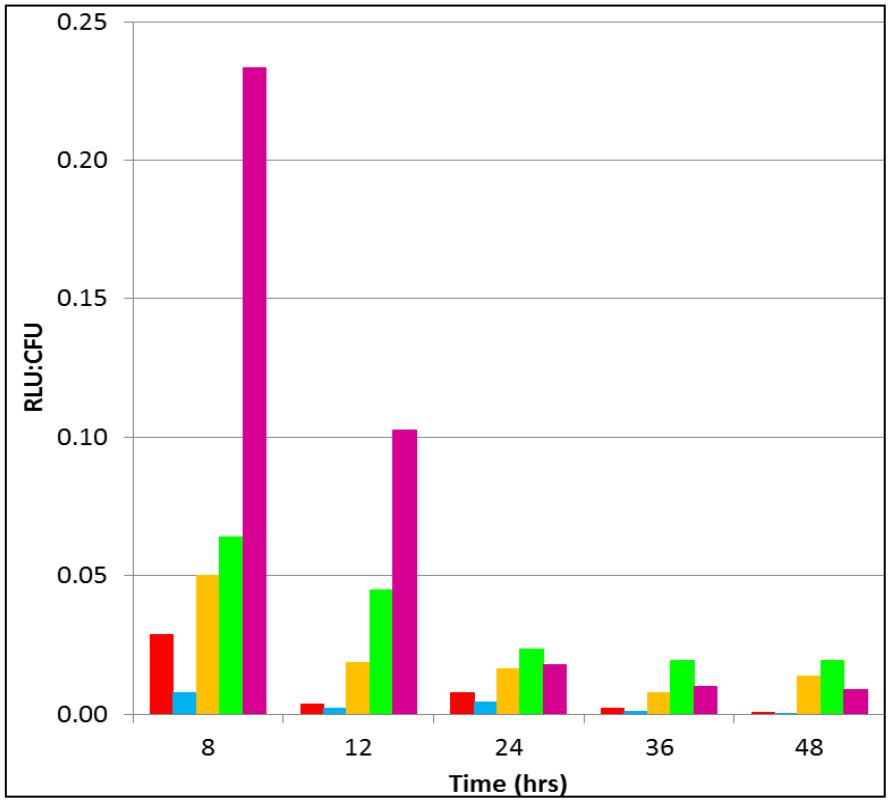


Figure 4.4: Relative promoter strength (RLU/CFU) during exponential and stationary phases. Bioluminescence and CFU count were measured at various time points along the growth curve and relative bioluminescence produced by a colony forming unit was calculated and is represented as a ratio of RLU to CFU. Relative promoter strength at various time points is shown. ldcC, spc, lys, tat, lpp.

Each promoter sequence was analysed for the -10 and -35 promoter sequences and the distance of the spacer regions (Table 4.1). This was related to the promoter strength for each bioluminescent (Figure 4.4).

Table 4.1: Promoter sequence comparison

	-10	-35	Spacer region
Consensus	*****TATAAT**	**TTGACA**	**17**
P_{tat}	TGG CACACT	TAGCCA	14
P_{lolB}	TGT CATGAT	TGGGCT	14
P_{lysS}	GGG CATTGT	TTTCCT	16
P_{ldcC}	ATG TATAAA	CTGCTG	13
P_{spc}	CGA TAAATT	TTTCAG	19

Red letters indicate differences from the consensus sequence.

It was shown that the -35 region of P_{spc} contained the most differences (4 out of 6bp) compared to consensus sequence (TTGACA) in *P. aeruginosa* and also the spacer region was 19 bp as opposed to 17bp. These differences may therefore relate to P_{spc} being the weakest promoter of the five. Similarly, P_{ldcC} also contained 4 out of 6bp differences in the -35 region compared to the consensus sequence. However, the spacer region was found to be 13bp. The -10 regions of P_{spc} and P_{ldcC} indicated 2bp and 1bp differences, respectively, from the consensus sequence. This may explain why P_{ldcC} was the stronger promoter of the two.

P_{lys} was found to contain 3bp differences from the consensus sequence in both -10 and -35 regions. The spacer region was found to be 16bp, closest to the consensus spacing. P_{lpp} and P_{tat} both contain spacer regions of 14bp. However, P_{lpp} has 3bp differences from the consensus sequence in the -35 region and 2bp differences in the -10 region. P_{tat} contains 2bp differences from the consensus sequence in the -35 region and 3bp differences in the -10 region. Comparing the promoter strength to sequences for these three promoters, both P_{tat} and P_{lys} have 3bp differences from the consensus sequence in the -10 region and their strengths are similar during the exponential phase. P_{lpp} has the highest expression during exponential phase as it contains only 2bp differences from the consensus sequence in the -10 region as opposed to 3bp differences in the same region for P_{tat} and P_{lys}.

P_{lpp} and P_{lys} have 3bp differences from the consensus sequence in the -35 region. On the other hand P_{tat} has 2bp differences from the consensus sequence in the -35 region. Comparing the promoter strength to sequences for these three promoters, both P_{lpp} and P_{lys} contain 3bp differences from the consensus sequence in the -35 region and their strengths are similar during stationary phase. P_{tat} has shown the most constitutive expression during stationary phase since it has only 2bp differences in the -35 region as opposed to 3bp differences in the same region for P_{lpp} and P_{lys}.

4.4.3 Relationship between RLU, CFU and RFU

Pearson's correlations were performed for bioluminescence readings and CFU counts (Table 4.2). Strong correlations coefficients ($r > 0.91$) were observed for all recombinant constructs up to 24 hours and the correlations were statistically significant ($P<0.01$). The correlations were significantly weaker for ldcC and spc strains up to 48 hours; however significant strong correlations were maintained for lys, tat and lpp strains, with tat showing the strongest correlation ($r > 0.98$). Throughout the 28 days thereafter, correlations remained weak for ldcC and spc, while stronger correlations ($r > 0.6$) were observed for lys and lpp ($P<0.01$). Strong correlation coefficients ($r > 0.95$) were obtained for tat ($P<0.01$).

The decrease in correlation between bioluminescence and CFU after 48 hours led to further investigations of the state of recombinant cells. LIVE/DEAD BacLight stains, comprising Syto-9 and propidium iodide fluorescent dyes, were utilised to differentiate cells between live and dead bacterial cells along the growth and death curve. Fluorescence output was measured as relative fluorescence unit (RFU) produced by bacteria stained with these two dyes. As exemplified in Figure 4.5 and Figure 7.13 in Appendix 9, for all strains tested, it was shown that RFU readings increased proportionally as CFU count and RLU increased until 48 hours. After 48 hours, RFU readings remained relatively stable. However, CFU

count and RLU decreased greatly. Moreover, the difference between live cells RFU and total cells RFU remained insignificant, indicating that the cells were in a live state.

Correlations were strong between RFU and CFU ($r > 0.91$) up to 7 days for recombinant constructs and up to 28 days for wild-type and promoterless strains. These correlations were found to be statistically significant ($P<0.01$). However, the correlations between RFU and CFU decreased for up to 28 days for ldcC, spc and lpp strains. These correlations were not significant and therefore there was a clear difference between the CFU and RFU profiles for these strains. Strains lys and tat showed significant correlations ($r > 0.6$) between CFU and RFU at 28 days.

Correlations were strong between RLU and RFU ($r > 0.96$) and statistically significant ($P<0.01$) up to 24 hours for all constructs tested. Correlations decreased for ldcC and spc at 14 and 21 days, respectively. These correlations were found to be insignificant, thereby indicating a clear difference between the RLU and RFU profiles for these two strains. However, the correlations significantly improved ($P<0.05$) after 14 and 21 days respectively for ldcC ($r > 0.77$) and spc ($r > 0.64$). Correlations decresed significantly to $r > 0.6$ at 7 days for lys and tat, after which the correlations significantly increased to $r > 0.75$ up to 28 days. Correlations of lpp remained strong ($r > 0.83$) and significant throughout the 28 days.

Figure 4.5 and Figure 7.13 in Appendix 9 shows that the rate of decline in CFU count and RLU compared to RFU was much less for wild-type than for recombinant strains. The promoterless strain also showed a decline in CFU count and RLU compared to the RFU. However, the reduction was not as rapid as with the recombinant strains. Strong correlations ($r > 0.91$) were observed between CFU and RFU for wild-type and promoterless strains throughout 28 days.

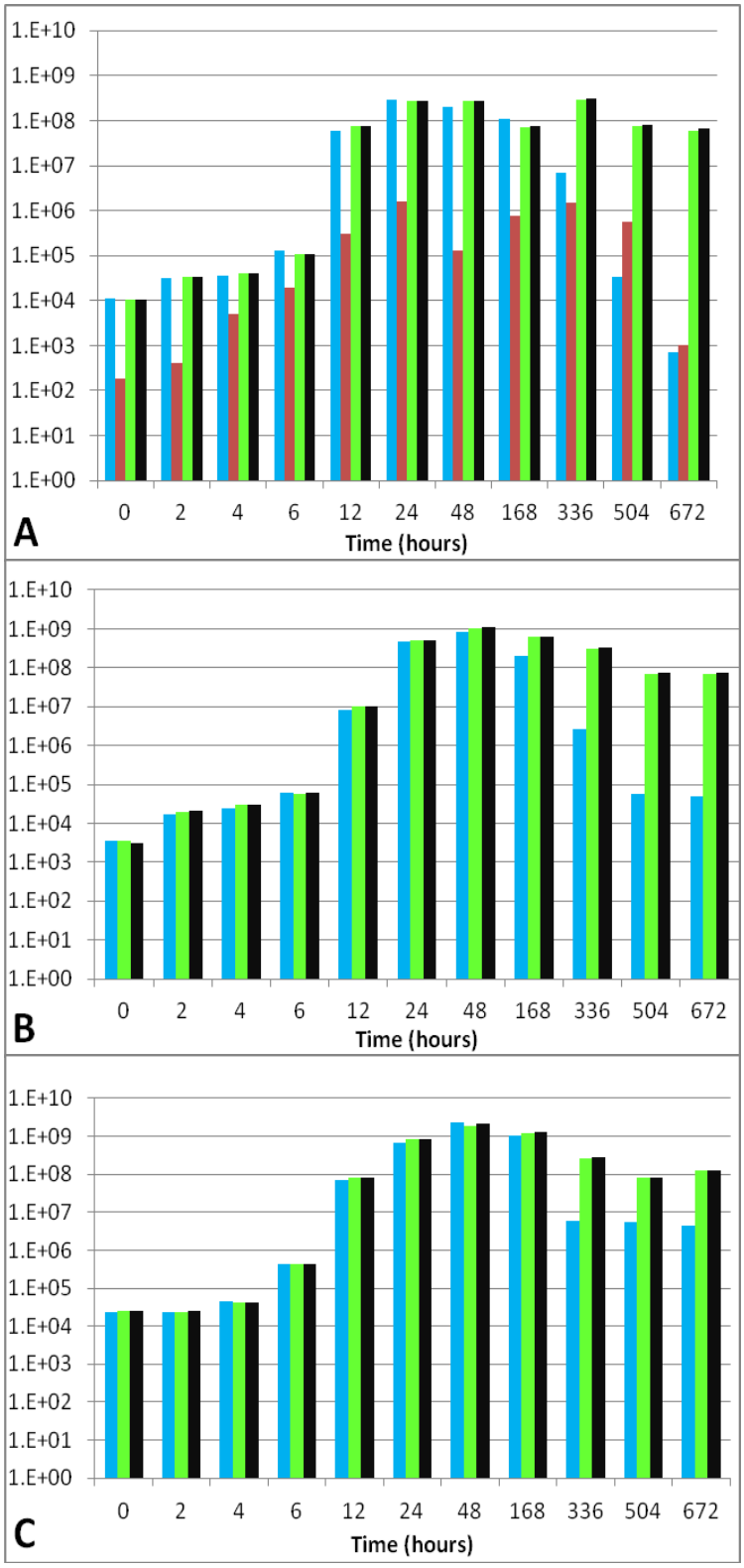


Figure 4.5: Comparisons of bioluminescence (RLU), colony forming units (CFU), live fluorescence (RFU) and total RFU at various time points. Time in hours is represented on X-axis of all graphs. CFU, RLU, Live RFU and Total RFU. A: lysS, B: pME-*lux*, C: WT.

Table 4.2: Pearson's correlations and significance between CFU, RLU and RFU at various time points for strains used for growth curve studies.

		ldcC	Spc	lysR25	lppR4	tatH5	pME*lux*	WT
CFU:RLU	**24hrs**	0.918** (0.000)	0.940** (0.000)	0.996** (0.000)	0.951** (0.000)	0.997** (0.000)		
	48hrs	0.375** (0.008)	0.358* (0.016)	0.848** (0.000)	0.773** (0.000)	0.987** (0.000)		
	7 days	0.376** (0.007)	0.360* (0.014)	0.843** (0.000)	0.744** (0.000)	0.986** (0.000)		
	14 days	0.225 (0.113)	0.289* (0.049)	0.624** (0.000)	0.622** (0.000)	0.959** (0.000)		
	21 days	0.214 (0.128)	0.289* (0.046)	0.600** (0.000)	0.608** (0.000)	0.961** (0.000)		
	28 days	0.235 (0.090)	0.313* (0.029)	0.615** (0.000)	0.623** (0.000)	0.962** (0.000)		
CFU:RFU	**24hrs**	0.996** (0.000)	0.995** (0.000)	0.998** (0.000)	1.000** (0.000)	1.000** (0.000)	1.000** (0.000)	1.000** (0.000)
	48hrs	0.972** (0.000)	0.992** (0.000)	0.980** (0.000)	0.998** (0.000)	0.992** (0.000)	0.997** (0.000)	0.996** (0.000)
	7 days	0.965** (0.000)	0.990** (0.000)	0.972** (0.000)	0.949** (0.000)	0.976** (0.000)	0.939** (0.001)	0.989** (0.000)
	14 days	0.461 (0.212)	0.548 (0.127)	0.652 (0.057)	0.580 (0.102)	0.606 (0.084)	0.910** (0.001)	0.983** (0.000)
	21 days	0.451 (0.191)	0.541 (0.106)	0.652* (0.041)	0.582 (0.078)	0.610 (0.061)	0.913** (0.000)	0.984** (0.000)
	28 days	0.459 (0.156)	0.541 (0.085)	0.657* (0.028)	0.587 (0.057)	0.617* (0.043)	0.915** (0.000)	0.984** (0.000)
RLU:RFU	**24hrs**	0.995** (0.000)	0.991** (0.000)	0.997** (0.000)	0.976** (0.001)	0.966** (0.002)		
	48hrs	0.237 (0.608)	0.200 (0.667)	0.688 (0.088)	0.836* (0.019)	0.626 (0.133)		
	7 days	0.284 (0.496)	0.224 (0.595)	0.650 (0.081)	0.834* (0.010)	0.630 (0.094)		
	14 days	0.783* (0.013)	0.662 (0.052)	0.765* (0.016)	0.908** (0.001)	0.803** (0.009)		
	21 days	0.775** (0.009)	0.648* (0.043)	0.759* (0.011)	0.908** (0.000)	0.802** (0.005)		
	28 days	0.776** (0.005)	0.640* (0.034)	0.758** (0.007)	0.899** (0.000)	0.804** (0.003)		

**. Correlation is significant at the 0.01 level (2-tailed), *. Correlation is significant at the 0.05 level (2-tailed). Significance values are shown in brackets.

Equivalence between the three methods was determined for each bioluminescent strain by performing analysis of variance at a significance level of P<0.05 (Table 4.3). No significant differences were found between the bioluminescent and plate count methods or between fluorescence and plate count methods since values were greater than 0.05. Significant differences were found between bioluminescence and fluorescence for bioluminescent strains except spc since values were less than 0.05.

Table 4.3: Equivalence between bioluminescence, CFU count and fluorescence.

	ANOVA (P<0.05)		
Strain	**RLU:CFU**	**RLU:RFU**	**CFU:RFU**
ldcC	0.572	0.017*	0.148
spc	0.392	0.052	0.508
lys R25	0.265	0.033*	0.547
lpp R4	0.464	0.034*	0.332
tat H5	0.525	0.023*	0.219

* P-values less than 0.05 indicate significant difference between bioluminescence and fluorescence.

4.5 Discussion

Bacterial growth and survival has been studied numerous times and has been shown to follow the standard pattern of five stages under favourable conditions. The aim of this study was to determine the growth and death patterns of all the bioluminescent strains produced and to compare them with those of wild-type *P. aeruginosa* ATCC 9027. Moreover, the bioluminescence method was compared to the traditional plate counting method to determine whether the bioluminescence method may be used as a determinant of bacterial growth and death kinetics.

4.5.1 Growth and death kinetics of *P. aeruginosa* wild-type and bioluminescent constructs

Wild-type and promoterless strains of *P. aeruginosa* were shown to grow up to 10^9 CFU/mL, whereas all constructs with promoters grew up to 10^8 CFU/mL. This may indicate a metabolic load of the gene expression of the *lux* cassette onto bacterial cells. It is unlikely that there could be a genetic load because the difference in size between the promoterless pME*lux* plasmid and promoter-*lux* plasmids was approximately 200bp. However, the luciferase pathway is highly energy-dependent and requires the essential cofactors NADPH, $FMNH_2$ and ATP. Moreover, the luciferase pathway has been shown to utilise myristol-ACP as the lipid substrate (Figure 4.6) (Close et al., 2012). Myristol-ACP is also essential for membrane lipid formation. Hence, there may be competition for the requirement of myristol-ACP between membrane lipid formation and luciferase pathway. Furthermore, myristol-ACP may also be utilised in the luciferase pathway at the expense of membrane formation, thereby reducing the formation of cell membranes of bioluminescent strains compared to the wild-type. This phenomenon of bioluminescence taking precedence has also been shown previously (Zarubin et al., 2012). This hypothesis is supported by the growth of the promotorless strain. The

promoterless strain contains the *lux* cassette. However, its transcription was minimal; therefore, the growth rate of the promoterless strain was similar to that of the wild-type because myristol-ACP was utilised for membrane formation rather than in the luciferase pathway.

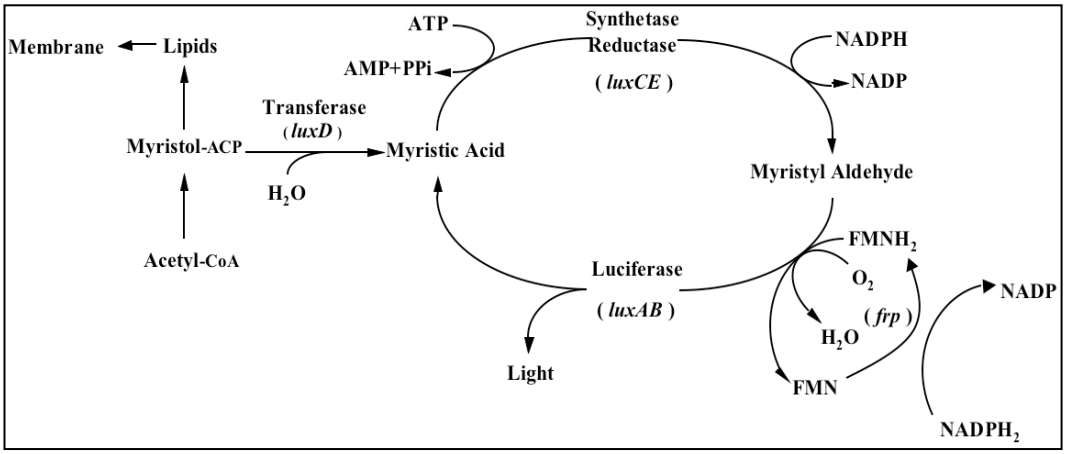


Figure 4.6: Schematic diagram showing competitive distribution of myristol-ACP (Close et al., 2012).

The lag phase lasted for approximately 3 hours. This was observed as bacterial cell counts remained constant for the first four hours upon inoculation into growth medium. However, there was an increase in bioluminescence during the lag phase, suggesting that increased constitutive gene expression was occurring during the lag phase with cells showing increased transcription rates for protein synthesis (Al-Qadiri et al., 2008) and replication of genomic and plasmid DNA to prepare the cells for cell division. Also, increase in bioluminescence per cell showed that there was increase in number of plasmids per cell as the cell increased resources before cell division into daughter cells. Hence bioluminescent constructs showed increase in gene expression at constant bacterial counts. Peaking on the RLU:CFU plot (Figure 4.3) were observed for all promoter containing constructs during the lag phase, indicating increased gene expression per bacterial cell. This aspect of bioluminescence detection prior to cell replication may provide a useful tool in detection of early microbial growth.

Following the lag phase, all recombinant constructs and the negative control strains entered the exponential growth phase. This phase lasted from approximately 3 hours to 12 hours. Increase in constitutive gene expression was proportional to CFU as cells replicated exponentially. This showed that there was constant gene expression as the cells replicated constantly (Al-Qadiri et al., 2008). Strong correlations between RLU and CFU count were also shown by Thorn *et al.* (2007). Tailing of the peaks on the RLU:CFU plot were observed as the gene expression per bacterial cell count stabilised to linear relationships. This means that the increased gene expression per CFU count, as observed during the lag phase, reduced so that consistent gene expression was observed with cell replication.

The onset of the stationary phase was observed at 12 hours. There was no net increase or decrease in bioluminescence or bacterial cell count. From the RLU:CFU plot, it was observed that constant light was produced per bacterial cell. Bioluminescence was observed to decline during the stationary phases of ldcC and spc strains. This may have been due to lower or no binding affinities of other sigma factors during the stationary phase. Hence transcription initiation from these two promoters would reduce during stationary phase. P_{spc} is also a binding site for soxS, as revealed by Softberry analysis. SoxS proteins are activator proteins that bind to the soxbox sequences (Wood et al., 1999). For the strains with weak promoters, that is ldcC and spc, strong correlations between CFU and Relative Fluorescence Units (RFU) indicative of cell viability were observed between 48 hours and seven days. But there were weaker correlations between bioluminescence and CFU or RFU. This indicated that cells maintained the ability to culture but were unable to utilise the luciferase pathway. This may have been due to their promoters being less recognised by the stationary phase sigma factors called sigma-38. Other studies have shown that *P. aeruginosa* sigma-38 is predominant during stationary phase (Raivio & Silhavy, 2001).

After 14 days, bioluminescence production was not proportional to bacterial cell count. Bioluminescence was observed to increase while bacterial count decreased, thereby indicating that gene expression was increasing but bacterial cells did not replicate. This inconsistency between bioluminescence and CFU count may indicate that bacterial cells are able to illuminate and are therefore viable; however, these are non-culturable on agar media. This was further confirmed when differences were obtained between the bioluminescence method and fluorescence method for all bioluminescent strains except spc. Fluorescence detection with the aid of Molecular Probes® Live/Dead® BacLight™ staining comprising of two dyes Syto-9 and propidium iodide (Berney, Hammes, Bosshard, Weilenmann & Egli, 2007; Molecular Probes) discriminated between viable and non-viable live cells. These two dyes are DNA intercalators (Berney et al., 2007); Syto-9 crosses intact membranes of viable cells and fluoresces green whereas Propidium iodide is able to cross damaged membranes only (non-viable cells) and fluoresces red. Hence, viable cells and non-viable cells can be distinguished by their fluorescence output. This method of estimating viable and non-viable cells is a rapid method that has been used in numerous studies (Ferreira, Pereira, Pereira, Melo & Simoes, 2011; Harrison et al., 2008). Investigations showed that cells remained fluorescent throughout 28 days. With the loss in culturability, bioluminescent constructs seemed to be entering a state of VBNC after 7 days. Furthermore, reduction in bioluminescence also suggested reduced gene expression, which is another characteristic of possible VBNC state (Navarro Llorens et al., 2010). VBNC state is a long-term stationary phenotype, which is characterised by cell death and survivors scavenging dead cell metabolites. Therefore there is a dynamic pattern of cell death and cell growth. However, the total number of cells remains the same. In such states, bioluminescence is observed to be reduced as cells divert their energy usage towards adaptation and growth in metabolite-rich environments rather than the expensive luciferase pathway. Such observation is a characteristic of the GASP (Growth Advantage in Stationary Phase) phenotype described in previous studies (Navarro

Llorens et al., 2010). Conversion of phase from stationary to long-term stationary phase seems to occur between 7 and 14 days. This has been indicated by the fact that correlations are strong between fluorescence and bioluminescence but weaker between fluorescence and culturable cells.

Over the next 14 days the bioluminescence decreased with further reduction in bacterial count. Wild-type CFU count decreased up to 14 days. However, the CFU count of the promoterless strain decreased up to 21 days, after which the CFU counts were stable. It has been shown that the inability of the bioluminescent strains to survive as well as wild-type and promoterless strains may be due to the metabolic load they carry. The metabolic load on the bioluminescent constructs may also play a crucial role in driving cells towards VBNC states when conditions were stressful so that bioluminescent cells did not have the capability to replicate. Bacterial cells are robust cells able to survive ever-changing environments. Survival strategies include control of transcription and translation to minimise energy wastage as a result of excess synthesis of mRNA and proteins (Neilson et al., 1999).

For the strains with strong promoters, that is lys, lpp and tat, strong correlations up to 14 days indicate that a highly significant number of cells were viable, expressing the *lux* cassette and were culturable. Strong correlations between bioluminescence and plate counting have also been observed previously (Thorn et al., 2007). For all recombinant strains with promoters, after 14 days strong correlations between RLU and RFU indicate that only viable cells are expressing the *lux* cassette. Statistical evaluation using ANOVA at $P<0.05$ found no significant difference between relative bioluminescence and CFU count and between CFU count and relative fluorescence reading.

4.5.2 Promoter strength and sequence analysis

Promoter strength of the five selected promoters was investigated by determining the bioluminescence rates per CFU. Promoter strength for all bioluminescent constructs was further related to their respective promoter sequences. During exponential phase, the promoter strength decreased in the order lpp>tat>lys>ldcC>spc while during stationary phase the promoter strength decreased in the order tat>lpp>lys>ldcC>spc, as indicated from the RLU:CFU plot. Weak expression of LdcC was also investigated by Lemonnier & Lane (1998). They suggested that it is regulated by a weak promoter and that low levels of the enzyme suggested that either transcription occured at a low level or the mRNA transcripts were unstable (Lemonnier & Lane, 1998).

The -35 region of P_{spc} contained the most differences from the consensus sequence and also the spacer region was longer than the 17bp observed in the consensus sequence. These differences may relate to P_{spc} being the weakest of the five promoters. P_{ldcC} also contained the most differences in the -35 region. However, the spacer region was found to be the smallest. Also the -10 regions of P_{spc} and P_{ldcC} have a 2bp and 1bp difference from the consensus. This may explain why P_{ldcC} was the stronger out of the two promoters. The importance of the spacer region was highlighted because P_{ldcC} and P_{spc} consist of spacer regions whose distances are at extremes of the consensus requirement. Hence, these two promoters were found to be the weakest of all five promoters investigated. Similar results have been reported previously where promoters containing spacer regions of 17bp have shown higher expression *in vitro* and *in vivo,* compared to those with longer or shorter spacer regions (Ayers et al., 1989; Mulligan, Brosius & McClure, 1985; Stefano & Gralla, 1982).

P_{lys} was found to contain 3bp differences from the consensus in both -10 and -35 regions. The spacer region was the closest to the consensus spacer. P_{lpp} and P_{tat} both contain spacer regions 3bp shorter than the

consensus spacer region. However, P_{lpp} has 3bp differences in the -35 region and 2bp differences in the -10 region. On the other hand P_{tat} has 2bp differences in the -35 region and 3bp differences in the -10 region. Comparing the promoter strength to sequences for these three promoters, both P_{tat} and P_{lys} have 3bp differences in the -10 region and their strengths are similar during exponential phase. P_{lpp} had the greatest level of expression during exponential phase as it contains only 2bp differences in the -10 region as opposed to 3bp differences in the same region for P_{tat} and P_{lys}. Outer membrane lipoprotein is one of the most abundant proteins (Inouye & Inouye, 1985) and therefore its expression controlled by P_{lpp} has been indicated by P_{lpp} showing the highest expression during exponential phase. Softberry analysis of promoter sequences revealed that P_{lpp} is also a binding site for rpoS18, a transcription binding factor (Yan, Lovley, & Krushkal, 2007). Sigma 38 (rpoS) is produced in response to stress and is active during stationary phase.

P_{lpp} and P_{lys} have 3bp differences in the -35 region. On the other hand, P_{tat} has 2bp differences in the -35 region. Comparing the promoter strength to sequences for these three promoters, both P_{lpp} and P_{lys} contain 3bp differences in -35 region and their strengths are similar during stationary phase. P_{tat} had relatively stable expression during stationary phase compared to that of other promoters and it has only 2bp differences in the -35 region as opposed to 3bp differences in the same region for P_{lpp} and P_{lys}. The transport of proteins to cytoplasmic membranes through the Tat pathway is vital (Jack et al., 2001) and therefore it is very important to maintain the expression of the tat proteins. Hence, it seems that the -10 region is more crucial for gene expression in the exponential phase while the -35 region is more crucial for gene expression during the stationary phase.

The most important elements of a promoter region are -10 region, -35 region and the spacer region in between them (DeHaseth et al., 1998). Various studies have shown that gene expression increases with increased similarity to the consensus sequences (Harley & Reynolds,

1987; Kammerer, Deuschle, Gentz & Bujard, 1986). Furthermore, the -10 and -35 promoter sequences are recognised by the σ subunit of RNA polymerase and therefore any differences in these regions would affect their gene expression (Harley & Reynolds, 1987). P_{tat} showed the smallest number of differences in the -35 region whereas P_{spc} and P_{ldcC} showed the most differences and therefore P_{tat} showed higher expression than P_{spc} and P_{ldcC}. Therefore, it would be expected that P_{ldcC} showed high gene expression. However, this was not the case as its spacer region was too small. Various studies have shown that the upstream half of the -10 region is more important to σ factors (Dombroski, 1997; Dombroski, Johnson, Lonetto & Gross, 1996; Dombroski, Walter, Record, Siegele & Gross, 1992); therefore the spacer region would cause a greater impact than regions downstream of the -10 region.

4.6 Conclusion

Increased gene expression per bacterial CFU for 3 hours showed the lag phase of bacterial growth. Exponential phase of bacterial cells was defined where the gene expression per bacterial cell count stabilised to linear relationships. The onset of the stationary phase was observed at 12 hours, where there was no net change in bioluminescence and bacterial cell counts, therefore consistent RLU:CFU was observed.

Relative promoter strength of the five promoters in *Pseudomonas aeruginosa* decreased in the order lpp>tat>lys>ldcC>spc during the exponential phase and in the order tat>lpp>lys>ldcC>spc during the stationary phase. P_{lpp} showed the greatest level of expression during the exponential phase while both P_{lpp} and P_{lys} showed similar strengths during the stationary phase. P_{tat} showed relatively stable expression during the stationary phase compared to all other promoters tested.

The bioluminescent method was equivalent to the traditional plate counting method since no statistically significant differences were observed. Correlations between bioluminescence, fluorescence and CFU count provide an indication of growth and death kinetics of the bacterial cells. Strong correlations between bioluminescence and CFU count indicated healthy metabolising and reproducing cells, while weak correlations between CFU and bioluminescence indicated that the cells were metabolising but unable to reproduce on media. A metabolic load of *lux* expression was observed on bacterial cells as bioluminescence was less at increased CFU count. Coupling of bioluminescence to bacterial metabolism with respect to constitutive gene expression provides a powerful, non-destructive tool for studies of bacterial growth during various growth phases in real time mode. Bioluminescence has been shown to give an earlier indicator of the health state of bacterial cells compared to traditional plate counting.

Chapter 5: Efficacies of benzalkonium chloride and benzyl alcohol against *P. aeruginosa* ATCC9027 constructs

5.1 Introduction

5.1.1 Benzalkonium chloride (BKC) and its characteristics

Benzalkonium chloride (BKC), classified as a quarternary ammonium compound (QAC), is a synthetic nitrogenous cationic antimicrobial agent with amphoteric surface-acting properties (Baudouin, Labbe, Liang, Pauly & Brignole-Baudouin, 2010; Fazlara & Ekhtelat, 2012). BKC consists of two hydrophobic hydrocarbon regions and a hydrophilic polar region (Figure 5.1) (Ferreira et al., 2011).

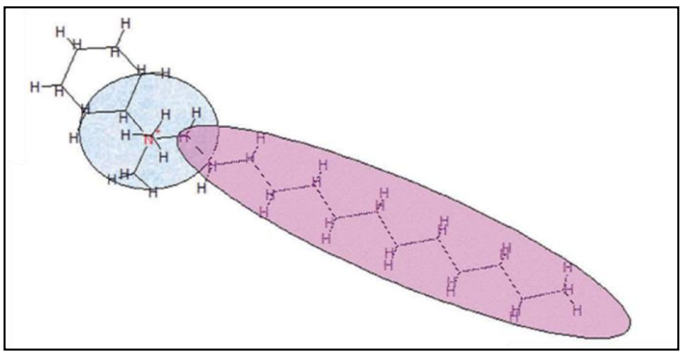


Figure 5.1: Structure of benzalkonium chloride (BKC) showing polar hydrophilic (blue) and non-polar hydrophobic region (purple). Adapted from Gilbert & Moore (2005).

These molecules are highly water-soluble (Fazlara & Ekhtelat, 2012). BKC is widely used in the food industry, hospitals and households as a disinfectant and antiseptic sanitizer (Fazlara & Ekhtelat, 2012). In pharmaceutical preparations, BKC is primarily used as a biocide or antimicrobial preservative in eyewashes, nasal sprays (Graf, 2001; Hodges, Denyer, Hanlon & Reynolds, 1996; Lenoir, Adriaens & Remon, 2011; Marple, Roland & Benninger, 2004), ophthalmic solutions (Mohanty, Acharya & Mishra, 2012; Rase, Bodkhe, Kshirsagar & Bedi, 2011; Ryan, Fain, Lovelace & Gelotte, 2011), amongst others. Interestingly, benzalkonium chloride is also used as an antimicrobial agent in acrylic carpets (Khajavi, Sattari & Ashjaran, 2007).

BKC is a broad-spectrum antimicrobial agent that possesses activity against both gram-positive and gram-negative bacteria as well as fungi and viruses. BKC cannot be classified as an antibiotic due to its lack of selectivity (McDonnell & Russell, 1999). BKC possesses both bacteriostatic and bactericidal properties.

There is increasing concern over BKC resistance, especially in *Pseudomonas aeruginosa*. Resistance of *Pseudomonas* to BKC was reported as early as 1969 (Adair, Geftic & Gelzer, 1969). One reason for this is because *P. aeruginosa* forms biofilms that provide a protective barrier, facilitate the formation of bacterial clusters and therefore provide bacteria with intrinsic resistance towards some antimicrobial agents. *Pseudomonas* is able to adapt to BKC, a feature known as antimicrobial tolerance, whereby BKC binds to the biofilm polymer and becomes ineffective. Increased synthesis of proteins involved in oxidative stress response, alginate synthesis and synthesis of cell membrane components have also been reported (Sheldon, 2005; Szomolay, Klapper, Dockery & Stewart, 2005). Although BKC is able to penetrate through the biofilm layers, *Pseudomonas* cells take advantage of the delay in penetration, thereby showing intrinsic resistance (Bridier, Dubois-Brissonnet, Greub, Thomas & Briandet, 2011). Another reason for intrinsic resistance is the expression of efflux pumps that are able to actively remove BKC back into the extracellular environment. Such resistance mechanisms provide one of the major reasons for persistent and repeated infections caused by *Pseudomonas.*

5.1.2 Mode of action of BKC

BKC is thought to change the hydrophobicity of bacterial cell membranes, thereby altering the cell membrane functions. Bacterial cell membranes carry a net negative charge on their surface, with cations stabilising the negative charge (Fazlara & Ekhtelat, 2012).

BKC, being cationic, mimics the effect of the stabilising membrane cations by binding tightly to bacterial cell membranes (Figure 5.2) (Fazlara & Ekhtelat, 2012; Gilbert & Moore, 2005). Furthermore, it has been shown that the hydrophobic region of BKC interacts with the hydrophobic region of the cell surface membrane while the hydrophilic region of BKC forms channels through the membrane leading to its permeabilisation (Ferreira et al., 2011; Gilbert & Moore, 2005). The cell membranes are damaged and the intracellular materials are liberated from the cells, thereby disrupting the cell homeostasis significantly. Formation of BKC pores also facilitates the entry of BKC molecules into the cells at high concentrations, coagulating the cytoplasm by its surfactant properties (Fazlara & Ekhtelat, 2012).

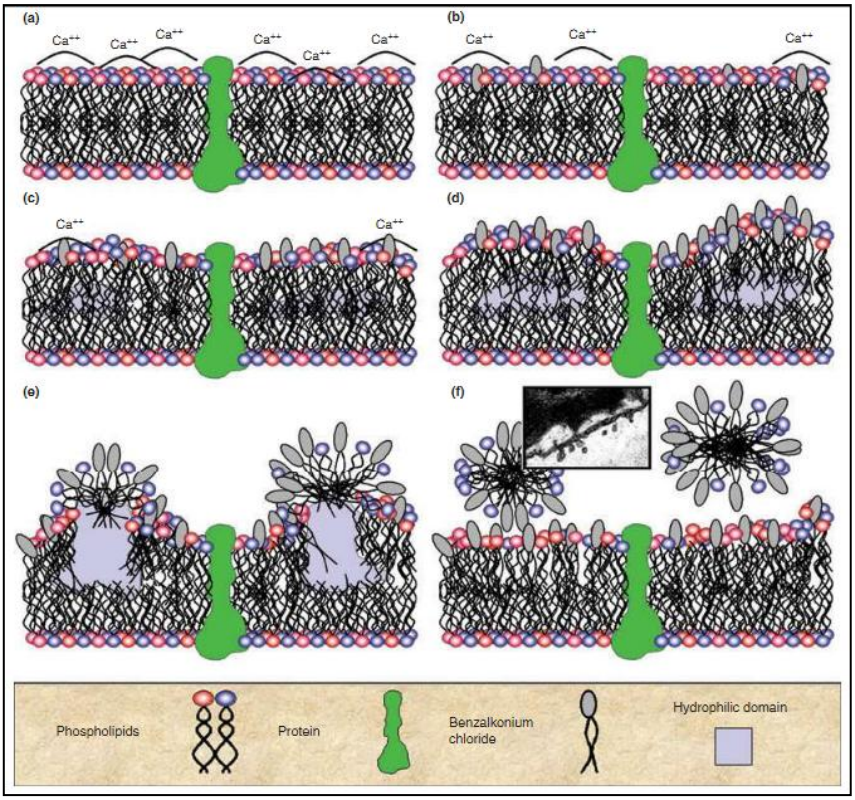


Figure 5.2: Mode of action of BKC. Steps a-f indicate the effect of increasing concentration of BKC on cell membranes leading to decreased fluidity and phospholipid micelle formation (Gilbert & Moore, 2005).

5.1.3 Benzyl alcohol (BA)

Benzyl alcohol is a volatile, cheap, non-flammable aromatic alcohol that is soluble in water up to a concentration of 4% ($^{v}/_{v}$) (GE healthcare, 2012). Benzyl alcohol is converted by oxidation to benzoic acid via the intermediate benzaldehyde. However, the reaction is very slow in aqueous solutions with no significant decrease in benzyl alcohol concentration for up to 3 years (GE healthcare, 2012). Interestingly, the activity of benzyl alcohol as a preserving agent is greatly reduced in the presence of polysorbate 80, rubber, polyethylene or polystyrene (Akers, 2002).

Benzyl alcohol is widely used as a preservative in pharmaceutical and cosmetic products. Benzyl alcohol is widely used in cosmetics, shampoos, toilet soaps and household cleaners, detergents (Scognamiglio, Jones, Vitale, Letizia & Api, 2012) and it remains one of the most commonly used preservative in multi-dose peptide and protein products (Meyer, Ni, Hu & Shi, 2007; Roy, Jung, Kerwin, Randolph & Carpenter, 2005). Benzyl alcohol is used as a volatile preservative for fruits and vegetables (Utama, Wills, Ben-Yehoshua & Kuek, 2002) and was found to have germicidal properties against *P. aeruginosa.* Heparin lock solutions used in hospitals for catheters also use benzyl alcohol as a preservative because of its convenience and low price (Shenep et al., 2011). However, following neonatal deaths its use has been abandoned in many hospitals (Gershanik, Boecler, Ensley, McCloskey & George, 1982). High concentrations of benzyl alcohol in cosmetic products are not permitted because they pose a high risk of allergic reactions. In fact, it is one of the most frequently reported allergens (Saiyasombati & Kasting, 2003).

5.1.4 Mode of action of BA

Benzyl alcohol is less effective against gram negative bacteria compared to gram positive bacteria and fungi (Meyer et al., 2007). Its mode of action against bacterial cells is not very well understood. However, it is thought that benzyl alcohol causes membrane damage and protein denaturation followed by metabolic interference and cell lysis (McDonnell & Russell, 1999). Benzyl alcohol is thought to interact with phosphatidylcholine vesicles, thereby altering membrane properties and organisation (Fishman, Rottem & Citri, 1980). At high concentrations of benzyl alcohol, proteins are denatured and form protein aggregates (Gupta & Kaisheva, 2003). It is thought that benzyl alcohol disrupts tertiary structures of proteins, thereby denaturing them (Lam, Patapoff & Nguyen, 1997).

5.1.5 Resistance to BA

Resistance to benzyl alcohol has been reported in *P. aeruginosa* (Singh, Kaur, Chakraborti, Jain & Banerjee, 2006). *Pseudomonas aeruginosa* cells have been shown to produce lipase in high amounts that transesterify benzyl alcohol to benzyl acetate. The lipase was shown to convert 90% of benzyl alcohol to benzyl acetate in 3 hours (Singh, Singh, Singh, Chisti & Banerjee, 2008).

5.2 Aim and objectives

The aim of this work was to assess the applicability of *Pseudomonas aeruginosa* ATCC 9027 and bioluminescent constructs for preservative efficacy tests with benzalkonium chloride and benzyl alcohol.

The objectives of this work were:

- To evaluate minimum inhibitory concentrations of BKC and BA.
- To compare preservative efficacy testing using the bioluminescence method, traditional plate counting method and fluorescence method.
- To evaluate five promoter-*lux* variants in a biosensor system for preservative efficacy testing.

5.3 Materials and methods

5.3.1 Bacterial strains

The *Pseudomonas* bacterial strains used for determining MIC and efficacy of BKC were described in section 3.3.1. The bacterial strains used for BA testing were:

Wild-type *P. aeruginosa* ATCC 9027 (WT)

P. aeruginosa ATCC 9027 + pME4510

P. aeruginosa ATCC 9027 + Promoterless pME-*lux*

P. aeruginosa ATCC 9027 + ldcC pME-*lux*

P. aeruginosa ATCC 9027 + spc pME-*lux*

P. aeruginosa ATCC 9027 + lys R25 pME-*lux*

P. aeruginosa ATCC 9027 + lpp R4 pME-*lux*

P. aeruginosa ATCC 9027 + tat H5 pME-*lux*

All strains were grown as described in section 3.3.1.

5.3.2 Minimum inhibitory concentration determination for BKC

Stock BKC (Sigma-Aldrich®) was prepared in sterile distilled water at a concentration of 5.12% ($^{w}/_{v}$) and pH of 7.0. The stock was filter-sterilised and stored at 20^{o}C in the dark. Working stocks of BKC were prepared at a concentration of 0.16% ($^{w}/_{v}$) in sterile distilled water. Various concentrations of BKC were then prepared in tryptic soya broth according to Table 5.1 for MIC determination. The volume of inoculum was set at 1% of total reaction volume in accordance with pharmacopoeial guidelines. Therefore, the inoculum volume used was 50 μL of cell culture for 5 mL total reaction volume.

Table 5.1: Concentration range of BKC used for MIC determination

Final BKC concentration (%)	**Volume of BKC (μL)**	**Volume of TSB (mL)**	**Volume of inoculum (μL)**	**Total volume (mL)**
0.04	1250	3.7	50	5
0.02	625	4.325	50	5
0.01	312.5	4.637	50	5
0.005	156.25	4.793	50	5
0.0025	78.125	4.871	50	5
0.00125	39.06	4.910	50	5
0.0006	19.53	4.930	50	5
0.0003	9.766	4.940	50	5
0	0	4.950	50	5

Each of the suspensions of BKC were prepared, in triplicate, in sterile cell culture plates and incubated for 24 hours at 32^{o}C and 100 rpm. The absorbance for each cell culture was measured at 600nm and recorded after incubation.

5.3.3 MIC determination for BA

Determination of BA (Sigma-Aldrich®) MIC was performed by preparing various concentrations of BA in tryptic soya broth according to Table 5.2. Undiluted BA was used for each preparation due to the limitation of solubility in water to prepare stock BA solutions.

Table 5.2: Concentration range of BA used for MIC determination

Final BA concentration (%)	**Volume of BA (μL)**	**Volume of TSB (mL)**	**Volume of inoculum (μL)**	**Total Volume (mL)**
2	100	4.850	50	5
1	50	4.900	50	5
0.5	25	4.925	50	5
0.25	12.5	4.938	50	5
0.125	6.25	4.944	50	5
0.0625	3.125	4.947	50	5

Each of the suspensions of BA were prepared, in triplicate, in sterile cell culture plates and incubated for 24 hours at 32^{o}C and 100 rpm. The absorbance for each cell culture was measured at 600nm and recorded after incubation.

5.3.4 Preservative efficacy test for BKC

BKC stock solution of 5.12% was prepared in sterile distilled water and filter-sterilised under aseptic conditions. BKC working solutions were prepared at various concentrations, ranging from 0.0003125% to 0.02%, in a total reaction volume of 100 mL using sterile distilled water as diluent as shown in Table 5.3.

Table 5.3: Concentration range of BKC used for PET determination

Working BKC concentration (%)	**Volume of stock (μL)**	**Volume of water (mL)**	**Volume of inoculum (mL)**	**Total volume (mL)**
0.02	4000	95	1	100
0.01	2000	97	1	100
0.005	1000	98	1	100
0.0025	500	98.5	1	100
0.00125	250	98.75	1	100
0.000625	125	98.875	1	100
0.0003125	62.5	98.9375	1	100
0	0	99	1	100

All strains were grown as described in section 3.3.1 Following incubation, aliquots (1 mL) of all cultures were centrifuged at 13000 rpm for 1 minute. The supernatant was discarded and the pellets were each re-suspended in 1 mL of sterile distilled water. This wash procedure was repeated once more before final re-suspension in sterile distilled water. Ten-fold dilutions were performed for WT, pME4510 and pME-*lux* strains in sterile distilled water.

For preservative efficacy testing, 1 mL of each of the washed (and in some cases diluted) cultures were inoculated aseptically into various concentrations of the preservative (Table 5.3) and incubated at 22^{o}C and 150 rpm for 28 days.

Samples were retrieved aseptically from each flask at various time points as per the preservative efficacy testing requirements: 0 hours, 6 hours, 24 hours, 7 days, 14 days, 21 days and 28 days. All samples were serially diluted 10-fold seven times in buffered peptone water (containing a mixture of peptides from partially hydrolysed proteins). Aliquots (1 mL) of each undiluted culture and diluted culture were transferred into culture tubes aseptically for bio-luminescence measurements as described in section 2.3.15. Aliquots (100 μL) of each undiluted and diluted culture were transferred onto TSB Agar plates as described in section 3.3.2. Samples were prepared for fluorescence quantification (section 4.3.2).

5.3.5 Preservative efficacy test for BA

BA working solutions were prepared at various concentrations, ranging from 0.0625% to 2%, in a total reaction volume of 100 mL using sterile distilled water as diluent (Table 5.4).

Table 5.4: Concentration range of BA used for preservative efficacy testing

Final BA concentration (%)	Volume of stock (μL)	Volume of inoculum (mL)	Volume of water (mL)	Total volume (mL)
2	2000	1	97	100
1	1000	1	98	100
0.5	500	1	98.5	100
0.25	250	1	98.75	100
0.125	125	1	98.875	100
0.0625	62.5	1	98.938	100

Preservative efficacy testing of benzyl alcohol was performed according to section 5.3.4.

5.3.6 Data analysis

Pearson's correlations between bioluminescence reading and plate count were performed at a significance level of $P<0.05$ for data obtained from each concentration of preservative.

Log unit reductions in plate count and relative bioluminescence were calculated as:

$$\frac{(\text{Log}_{10}\ \text{CFU at time}_{\ t}\ \text{and concentration}_{\ c}) - (\text{Log}_{10}\ \text{CFU at time}_{\ t0}\ \text{and concentration}_{\ c0})}{}$$

Equation 5.1: Log unit reduction

Two-way analysis of variance (ANOVA) at a significance level of $P<0.05$ was performed between bioluminescence and plate count data in order to determine the equivalence between these two methods.

5.4 Results

5.4.1 Minimum inhibitory concentration of BKC

The minimum inhibitory concentration of benzalkonium chloride was evaluated for all the strains by measuring absorbance at 600nm after challenge with a range of concentrations of BKC (0.0003% and 0.04%). Growth of wild-type, pME4510 and pME*lux* bacterial strains was observed in the negative controls (0% BKC), as well as dilute concentrations between 0.0003% and 0.005% (Table 5.5). The MIC of BKC for these three strains was therefore found to be 0.01%. The absorbance values obtained for wild-type, pME4510 and pME*lux* strains with 0% BKC were greater (>2.25) than those of the bioluminescent constructs (1.75). This was because these three strains grow up to 10^9 CFU/mL compared to the bioluminescent constructs that grow up to 10^8 CFU/mL, as discussed in section 4.5.1. However, for all bioluminescent constructs strains the MIC shifted to 0.00125%, whilst bacterial growth was observed at a BKC concentration of 0.0006%. Negative absorbance values were obtained for all strains tested at a BKC concentration of 0.04%. This concentration was too high and hence too toxic for bacterial cells. Physical observation of 0.04% BKC solutions indicated a cloudy appearance compared to all other solutions. This may also have had an effect on absorbance readings.

Table 5.5: Minimum inhibitory concentrations for BKC against all strains.

	Absorbance [a] at 600nm at various BKC concentrations (%)								
BKC conc (%)	**0**	**0.0003**	**0.0006**	**0.0013**	**0.0025**	**0.005**	**0.01**	**0.02**	**0.04**
WT	2.40	2.42	2.39	2.30	2.36	0.80	0.10	0.13	-0.01
pME4510	2.31	2.43	2.42	2.32	2.30	0.34	0.07	0.08	-0.08
pMElux	2.35	2.28	2.20	2.15	2.17	0.10	0.06	0.09	-0.05
ldcc	1.86	1.76	1.74	0.01	0.08	0.12	0.08	0.04	-0.01
spc	1.85	1.77	1.72	0.01	0.09	0.11	0.06	0.03	-0.01
lys R25	1.84	1.87	1.68	0.04	0.02	0.03	0.03	0.03	-0.10
lys G25	1.87	1.84	1.86	0.04	0.04	0.05	0.08	0.04	-0.02
lpp R3	1.88	1.86	1.86	0.03	0.03	0.03	0.03	0.04	-0.10
lpp R4	1.86	1.77	1.85	0.01	0.02	0.03	0.04	0.04	-0.03
tat H5	1.86	1.78	1.77	0.01	0.01	0.10	0.11	0.01	-0.05
tat H9	1.85	1.78	1.75	0.01	0	0.08	0.08	0.02	-0.07
tat H14	1.86	1.80	1.78	0.01	0.02	0.11	0.07	0.06	-0.07

[a] Absorbance was measured at 600nm.
Green indicates growth of *P. aeruginosa.* Red indicates no growth of *P. aeruginosa.*

5.4.2 Minimum inhibitory concentration of BA

The minimum inhibitory concentration of benzyl alcohol against all bacterial strains was evaluated by measuring absorbance at 600nm and bioluminescence of bacterial cells challenged with a range of concentrations of BA for 24 hours. The concentration range of the preservative tested was between 0.0625% and 2%. Growth for all bacterial strains was observed in dilute concentrations between 0.0625% and 0.125% (Table 5.6). However, the growth of all bacterial strains was reduced at BA concentrations of 0.25% and 0.5%. Absorbance and bioluminescence reduced greatly when bacterial strains were exposed to 1% or 2% benzyl alcohol. The MIC of BA for all strains was therefore found to be 1%. The absorbance values obtained for wild-type, pME4510 and pME*lux* strains with 0% BKC were higher compared to the bioluminescent constructs. This is because these three strains grow up to 10^9 CFU/mL compared to the bioluminescent constructs that grow up to 10^8 CFU/mL, as discussed in section 4.5.1.

Table 5.6: Minimum inhibitory concentration of BA against all strains.

	Relative bioluminescence (RLU)[a] at various BA concentrations (%)						Absorbance [b] at 600nm at various BA concentrations (%)					
Strain	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**	**2**
WT	197	214	218	212	244	275	2.452	2.437	1.931	0.103	0.030	0.055
pME4510	227	221	174	148	154	193	2.422	2.413	1.831	0.094	0.030	0.044
pMElux	149	149	137	137	144	156	2.330	2.367	1.730	0.050	0.031	0.048
ldcC	18225	20367	7495	4615	286	326	1.743	1.711	1.396	0.256	0.028	0.027
spc	17458	17329	5795	4692	180	209	1.785	1.728	1.408	0.266	0.022	0.029
lys R25	250841	236308	224372	75247	167	178	1.679	1.681	1.387	0.225	0.026	0.027
lys G25	408692	419256	235003	155118	218	246	1.674	1.654	1.319	0.179	0.026	0.027
lpp R3	113056	112845	69318	37068	194	184	1.678	1.665	1.368	0.214	0.025	0.030
lppR4	841075	624454	242494	199408	161	178	1.728	1.744	1.351	0.182	0.024	0.024
tat H5	357933	294127	162740	114918	132	139	1.729	1.747	1.325	0.231	0.026	0.027
tat H9	179145	213899	100449	48486	114	128	1.624	1.633	1.273	0.216	0.025	0.024
tat H14	83472	97596	63554	21869	155	152	1.571	1.608	1.290	0.188	0.022	0.020

[a] Relative bioluminescence was measured at 490nm.
[b] Absorbance was measured at 600nm.
Green indicates growth of *P. aeruginosa.* Red indicates no growth of *P. aeruginosa.*

5.4.3 Preservative efficacy test for BKC

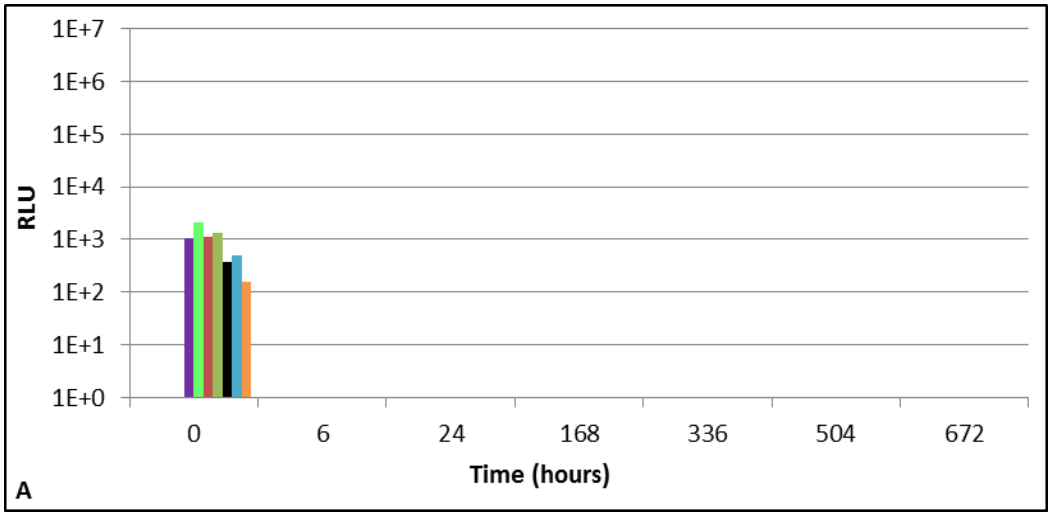
The effect of various concentrations of BKC was evaluated with all the strains exposed to preservative challenge. Bioluminescence, CFU count and fluorescence measurement were recorded at time intervals as defined by several pharmacopoeias. Wild-type *P. aeruginosa* and *P. aeruginosa* with either plasmid pME4510 or pME*lux* were used as negative controls for bioluminescence detection. Correlation coefficients and their significance are shown in Table 7.5 in Appendix 10. Log unit reductions in CFU and RLU are shown in Table 7.7 of Appendix 10. Equivalence between the bioluminescence method and traditional plate count method for BKC is shown in Table 7.9, Appendix 10.

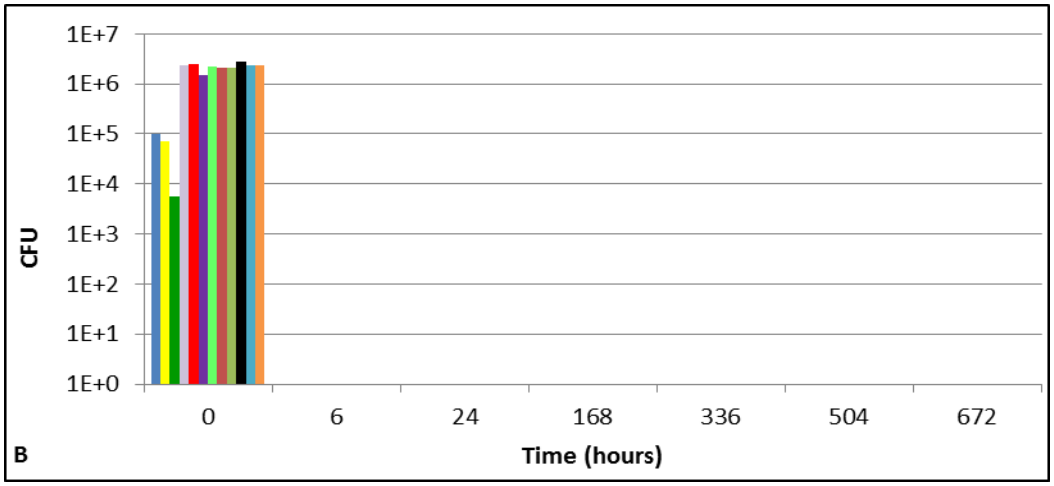
At high BKC concentrations of 0.02% and 0.01% (Appendix 10, Figure 7.14), negative control strains were 10^5 CFU/mL, bioluminescence was undetectable upon inoculation for bioluminescent constructs; however, CFU count in the range of 10^3 CFU/mL were observed for all

bioluminescent constructs. Hence, there was a 3 log reduction in CFU immediately upon inoculation. A further 3 log reduction in CFU was observed by 6 hours. It was therefore shown that bioluminescence was undetectable at such high concentrations of BKC upon inoculation of the strains with considerable reduction in CFU count. No CFU count was detected beyond time 0.

Bioluminescence was detected at 0 hour only for the strong promoters (lysR25 and lysG25) challenged with 0.005% and 0.0025% BKC (Appendix 10, Figure 7.14). No bioluminescence was detected at subsequent time points or for all other strains. For all strains, CFU count was 10^5 and 10^6 CFU/mL upon challenge with the same concentrations of BKC, respectively. No CFU count was detected beyond time 0. Correlations between bioluminescence and plate counts for lysR25 and lysG25 strains challenged with 0.005% BKC were found to be statistically significant ($P<0.05$) with correlation coefficients of 0.8 and 0.78 respectively. This was because lys was the strongest promoter out of the five promoters tested and therefore it produced higher levels of bioluminescence. At BKC concentrations of 0.0025%, correlations between bioluminescence and plate count were significant ($P<0.01$), with correlation coefficients greater than 0.9. Relative fluorescence (RFU) measurements were taken to determine viability of cells with respect to CFU counts. RFU readings were measured for strains challenged with 0.0025% BKC. RFU measurements for control strains and bioluminescent constructs were 10^5 and 10^6 RFU/mL, respectively, at only 0 hour time point. No further RFU readings were detectable for the subsequent time points.

Bioluminescence was detected at 0 hour for all constructs of lysS, lpp and tat (except ldcC and spc) challenged with 0.0125% BKC (Figure 5.3A). No bioluminescence was detected at subsequent time points or for all other strains. CFU counts were 10^5 and 10^6 CFU/mL for all control strains and bioluminescent strains, respectively (Figure 5.3B). No CFU count was detected after 0 hour. RFU measurements (Figure 5.3C) for control strains and bioluminescent strains were 10^5 and 10^6 RFU/mL, respectively, at time 0. No further RFU readings were detectable for the subsequent time points. Correlations between bioluminescence and CFU count, for all the strong promoter strains were significant ($P<0.01$) with correlation coefficients greater than 0.96. Viability testing of all the strains also resulted in significant correlations ($P<0.01$) between culturable cells and fluorescence, with correlation coefficients greater than 0.98. Correlations between bioluminescence and fluorescence were also significant ($P<0.01$), with coefficients greater than 0.96 for the bioluminescent constructs.





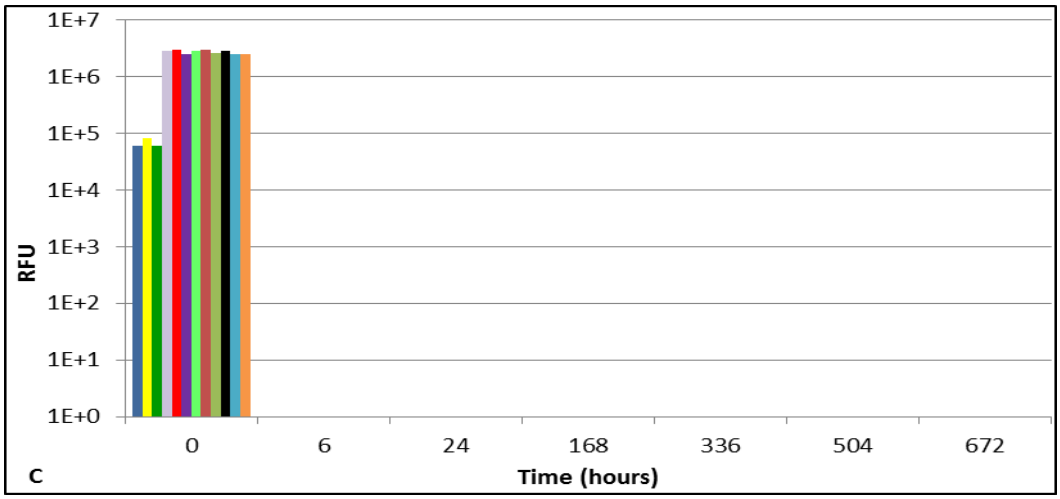


Figure 5.3: BKC efficacy test for all strains challenged with 0.00125% BKC.
Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

WT pME4510 pME*lux* ldcC spc lysR25 lysG25
lppR3 lppR4 tatH5 tatH9 tatH14

Bioluminescence was 10^3 and 10^4 RLU/mL for bioluminescent constructs with weak and strong promoters, respectively, when challenged with 0.0006% BKC (Figure 5.4A). Bioluminescence then reduced by 1 log unit at 6 hours for lysR25, lysG25, lppR3 and lppR4. No bioluminescence was detected up to 28 days for all other strains. CFU counts were greater than 10^5 and 10^6 CFU/mL, respectively, for all control strains and bioluminescent strains upon challenge with the same concentration of BKC (Figure 5.4B). CFU counts for bioluminescent constructs subsequently decreased to 10^4 CFU/mL at 6 hours and decreased further at 24 hours. No CFU counts were detected after 24 hours. RFU was measured for strains challenged with 0.0006% BKC (Figure 5.4C) and these were 10^5 and 10^6 RFU/mL for control strains and bioluminescent strains, respectively, at 0 hour. RFU for control strains subsequently decreased to 10^4 RFU/mL at 6 hours; however, for bioluminescent constructs RFU reduced to 10^4 RFU/mL at 24 hours. No further RFU readings were detectable for later time points. Correlations between bioluminescence and CFU count were significant ($P<0.01$) for all bioluminescent constructs with coefficients greater than 0.99. Correlations between bioluminescence and fluorescence were significant ($P<0.01$). However, the coefficients were slightly lower and ranged between 0.86 – 0.97 for all bioluminescent constructs. Furthermore, significant correlations ($P<0.01$) between CFU and RFU were obtained for all bioluminescent constructs except lysR25. However, the coefficients ranged between 0.85-0.97. Correlations for lysR25 were significant at $P<0.05$ with a coefficient of 0.85 indicating that not all cells were culturable. This was further confirmed when fluorescence was 10^4 RFU/mL at 6 and 24 hours but no CFU counts were detected for negative control strains at the same time points. A 2 log and 3 log reduction in CFU at 6 hours and 24 hours, respectively, was therefore observed for all bioluminescent strains. This related to a >2 log and 4 log reduction in bioluminescence at 6 hours and 24 hours, respectively.

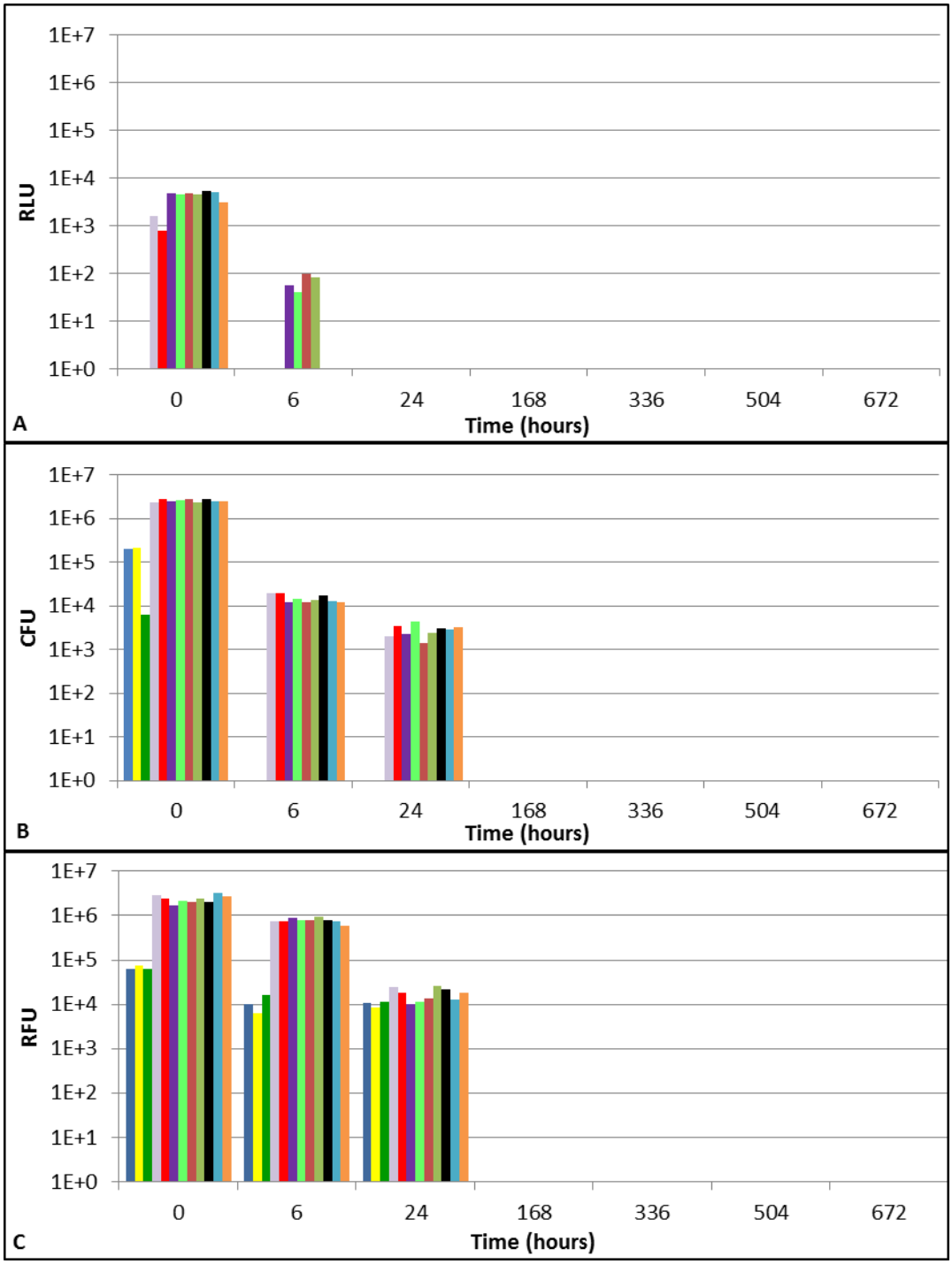


Figure 5.4: BKC efficacy test for all strains challenged with 0.000625% BKC. Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

WT pME4510 pME*lux* ldcC spc lysR25 lysG25 lppR3 lppR4 tatH5 tatH9 tatH14

Bioluminescence was 10^3 and 10^4 RLU/mL for bioluminescent constructs with weak and strong promoters, respectively, when challenged with 0.0003% BKC (Figure 5.5A). Bioluminescence decreased by a log unit for all promoters within 6 hours and by a further log unit for all promoters except ldcC and spc by 24 hours. No bioluminescence was detected from 24 hours up to 28 days for all strains. CFU counts were 10^6 CFU/mL (Figure 5.5B) for all strains at time 0. CFU counts for bioluminescent constructs decreased by a log unit by 24 hours and by 2 log units by 7 days. CFU counts for all bioluminescent strains except ldcC and spc were detected at 10^3 CFU/mL at 14 days followed by undetectable levels up to 28 days. No CFU counts were detected beyond 6 hours for the negative control strains. A similar pattern of fluorescence to CFU was found for all strains up to 24 hours. Relative fluorescence for negative control strains and bioluminescent strains (Figure 5.5C) declined even further to 10^3 RFU/mL and 10^4 RFU/mL, respectively, at 7 days. From 14 days onwards, negative control strains and ldcC and spc were undetectable. Strains lys, lpp and tat fluoresced consistently throughout the rest of the experiment. Correlations between bioluminescence, CFU count and fluorescence were significant ($P<0.01$). However, the correlations between bioluminescence and fluorescence were slightly reduced for all bioluminescent strains except tatH5 and tatH14. Correlations between culturable cells and fluorescence were also reduced slightly for lysR25, lysG25 and tatH14.

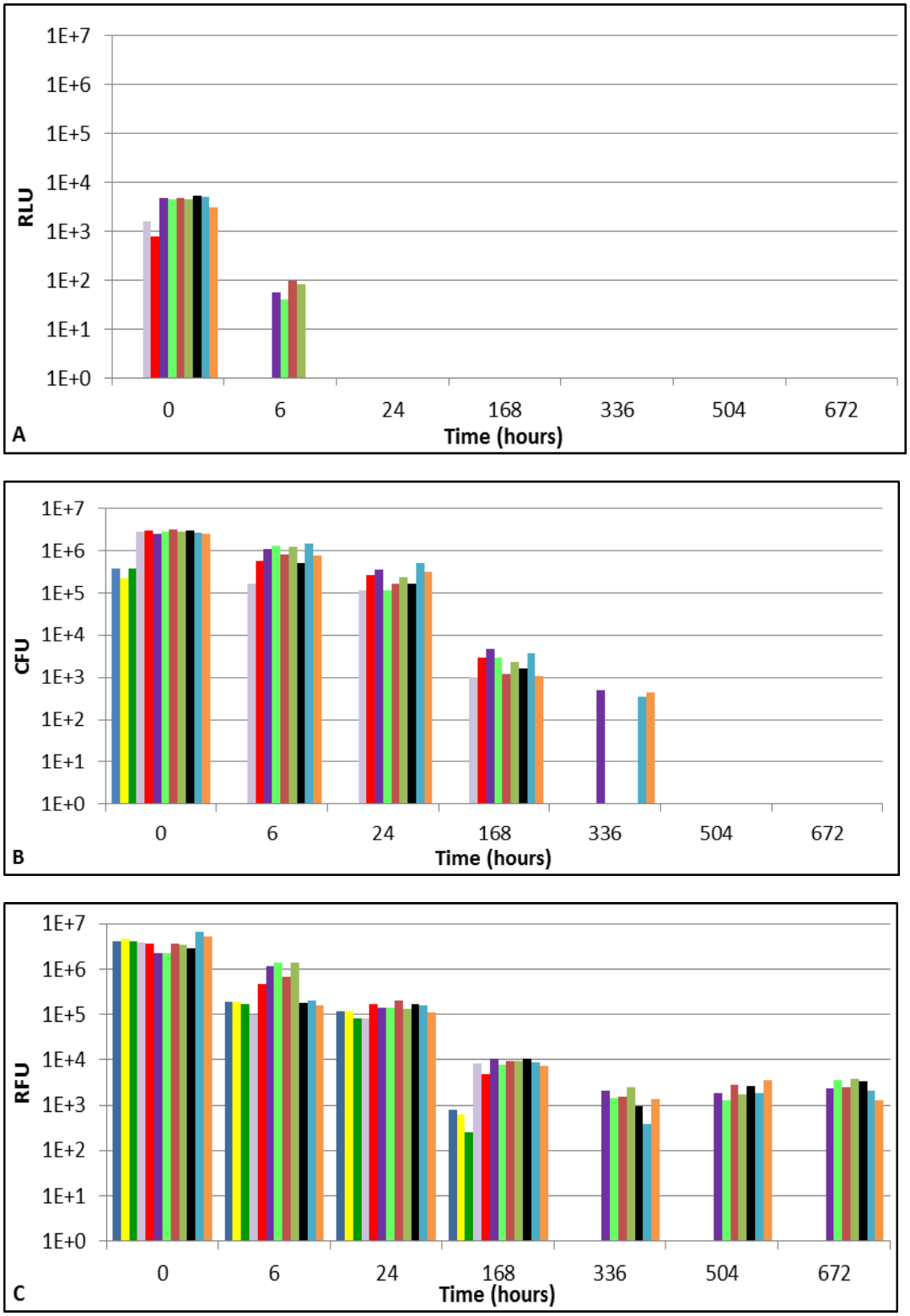


Figure 5.5: BKC efficacy test for all strains challenged with 0.0003125% BKC. Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

Correlations between CFU and RFU were significant ($P<0.01$) for negative control strains wild-type and pME4510 throughout 28 days when they were challenged with different concentrations of BKC. However, the correlations were slightly reduced to 0.89 and 0.93, respectively, when these strains were challenged with 0.0006% BKC. A similar pattern was found for the promoterless strain, where correlations were significant ($P<0.05$) and there was slight reduction in correlation coefficient to 0.85 with 0.0006% BKC challenge.

Bioluminescence was detected throughout 28 days for all promoter-*lux* constructs when challenged with 0% BKC (Figure 5.6A). CFU counts for all strains were approximately 10^6 CFU/mL and remained consistent throughout 28 days (Figure 5.6B). RFU measurements for all strains were 10^6 RFU/mL throughout 28 days and remained consistent (Figure 5.6C). The correlations between bioluminescence and CFU count were found to be weak and non-significant. However, correlations between culturable cells and fluorescence for all strains except ldcC, lysR25 and tatH14 were significant ($P<0.05$) with correlation coefficients above 0.8 when conditions were starved for 28 days.

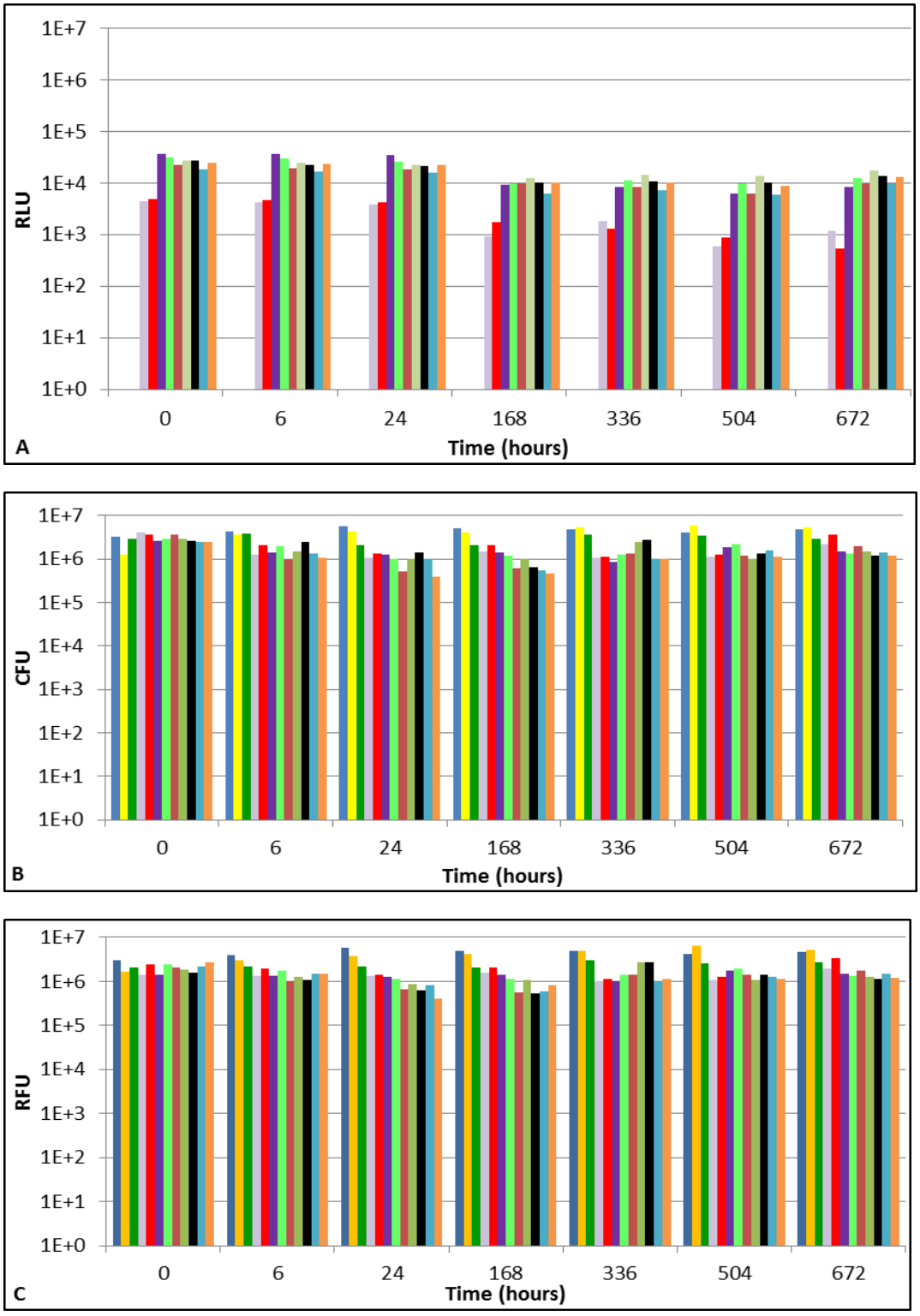


Figure 5.6: BKC efficacy test for all strains challenged with 0% BKC. Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

WT pME4510 pME*lux* ldcC spc lysR25 lysG25
lppR3 lppR4 tatH5 tatH9 tatH14

5.4.4 Preservative efficacy test for BA

The effect of various concentrations of BA was evaluated when the strains were exposed to preservative challenge. Bioluminescence, CFU count and fluorescence measurement were recorded at time intervals as specified by several pharmacopoeias. Correlations between bioluminescence, cell count and fluorescence are shown in Appendix 10, Table 7.6. Log unit reductions in CFU count and bioluminescence are shown in Table 7.8 of Appendix 10. Equivalence between the bioluminescence method and traditional plate count method for BA is shown in Table 7.9 of Appendix 10.

Wild-type *P. aeruginosa* and *P. aeruginosa* with either plasmid pME4510 or pME*lux* were used as negative controls for bioluminescence detection. At 2% BA, bioluminescence of 10^2 - 10^3 RLU/mL (Figure 5.7A), CFU count of 10^6 CFU/mL (Figure 5.7B) for bioluminescent constructs and 10^3 – 10^4 CFU/mL for negative controls and fluorescence of 10^6 RFU/mL (Figure 5.7C) were observed at 0 hours. A 2 log unit reduction in CFU was observed immediately after inoculation for the negative strains. Interestingly, fluorescence of 10^5 RFU/mL was also observed at 6 hours for all bioluminescent constructs, pME4510 and pME*lux* strains. However, no RLU and CFU counts were detected beyond 0 hour. Correlations between bioluminescence and CFU count and between bioluminescence and fluorescence were both significant ($P<0.05$) for all bioluminescent constructs except lysR25; however, they were lower than expected, with correlation coefficients ranging from 0.79 to 0.83.

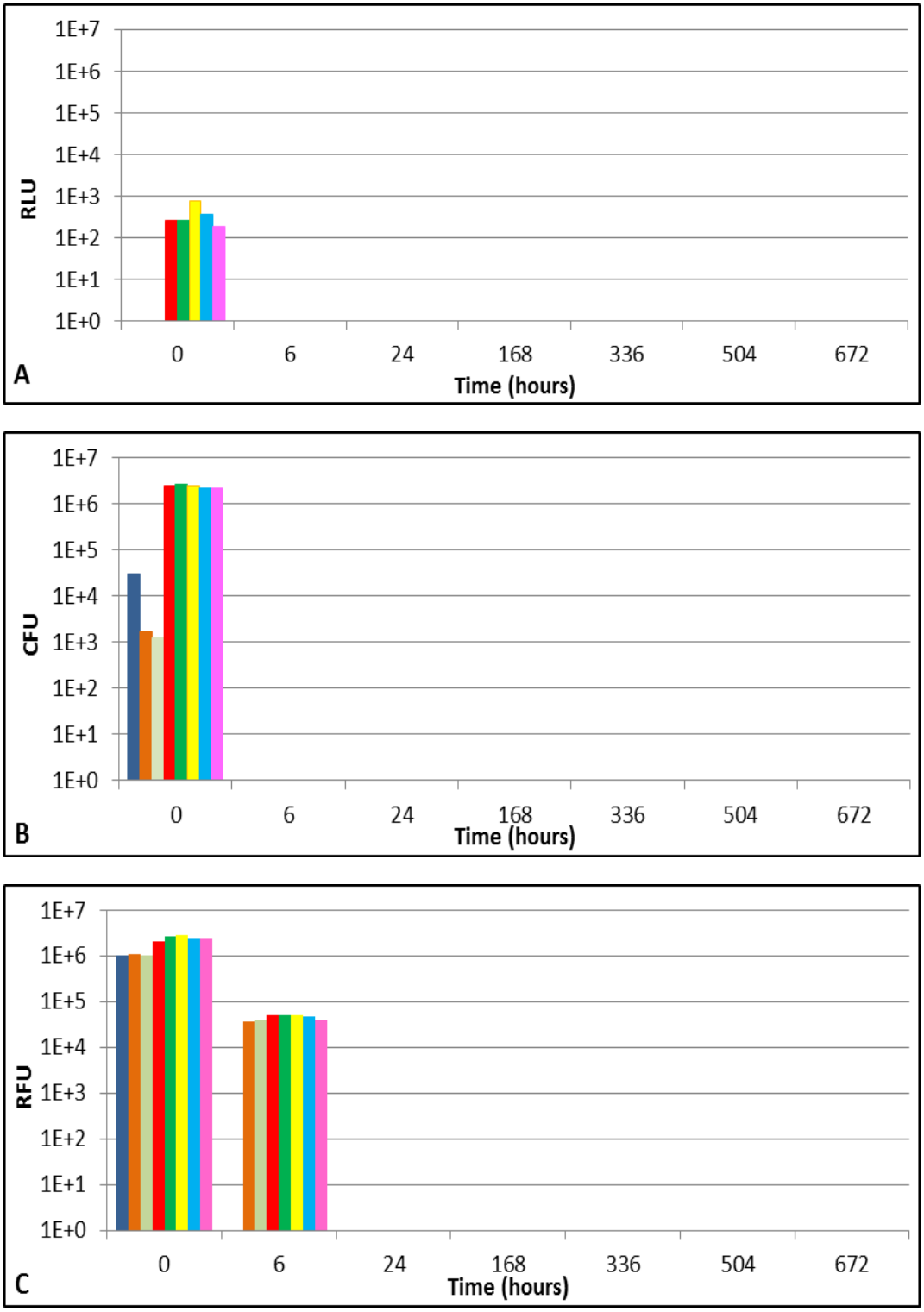


Figure 5.7: BA efficacy test for all strains challenged with 2% BA. Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

Bioluminescence of approximately 10^3 RLU/mL (Figure 5.8A) from all bioluminescent constructs was detected at 0 hours when challenged with 1% BA. Bioluminescence then reduced by a half log unit at 6 hours for all bioluminescent constructs and completely for spc. Bioluminescence reduced further by a half log unit at 24 hours and completely for ldcC. No bioluminescence was detected at subsequent time points or for all other strains. CFU counts for all strains were 10^6 CFU/mL after challenge with the same concentration of BA (Figure 5.8B). A slight reduction in CFU counts was observed at 6 and 24 hours for all bioluminescent strains. A 6 log unit reduction in CFU was observed at 6 hours for negative control strains and at 7 days for bioluminescent strains. Relative fluorescence for all strains was 10^6 RFU/mL after challenge with the same concentration of BA (Figure 5.8C). A slight reduction in RFU was observed at 6 and 24 hours for all strains. No RFU counts were detected after 24 hours. Correlations were significant ($P<0.01$) between bioluminescence, CFU count and fluorescence, with correlation coefficients greater than 0.92.

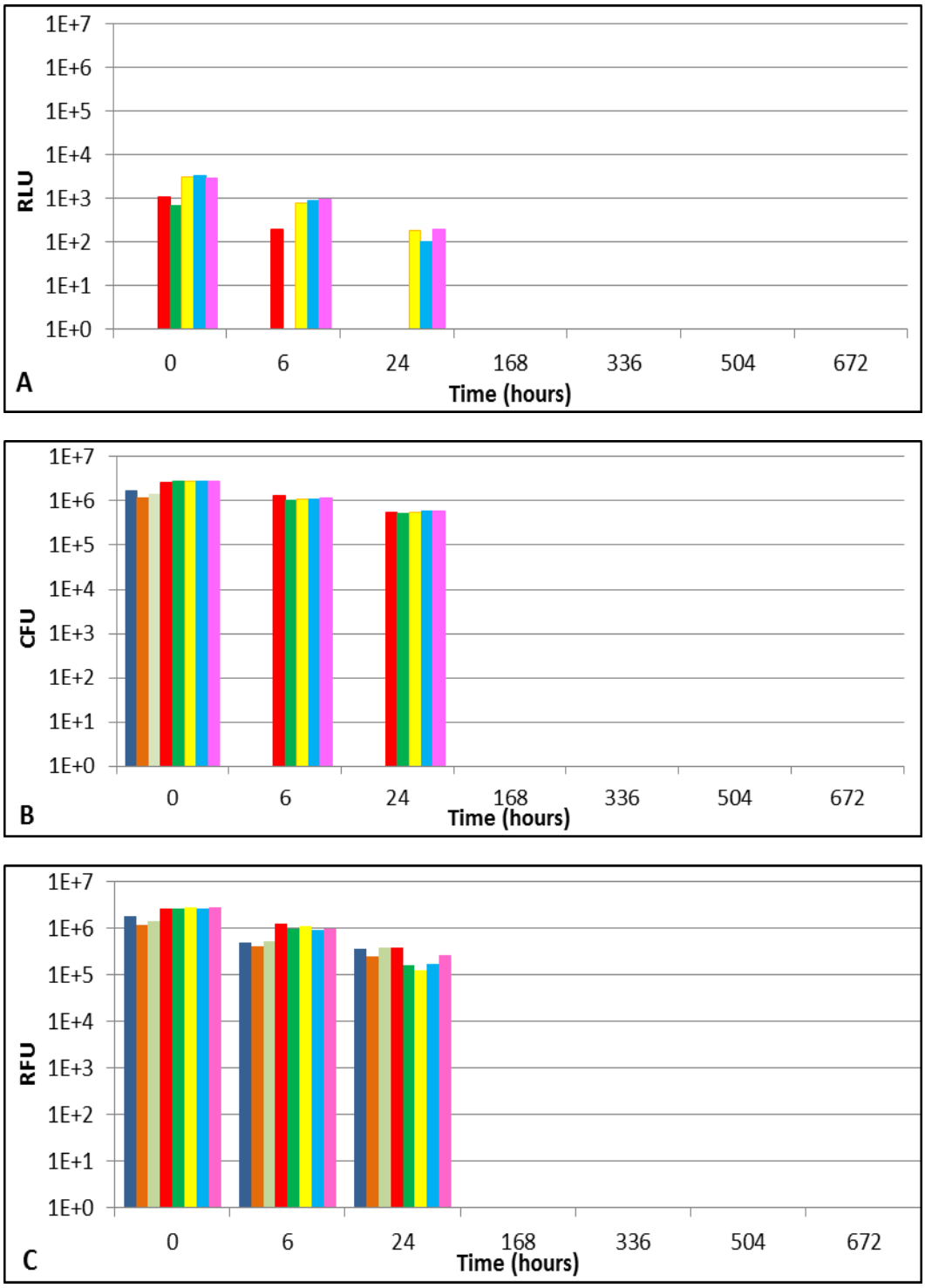


Figure 5.8: BA efficacy test for all strains challenged with 1% BA. Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

A gradual decrease in RLU from 10^4 RLU/mL (ldcC, spc) and 10^5 RLU/mL (lys, tat, lpp) respectively, to approximately 10^2 RLU/mL, within the first 7 days was observed for strains challenged with 0.5% BA (Figure 5.9A). Bioluminescence then remained consistent up to 28 days for bioluminescent strains with strong promoters. However, bioluminescence for ldcC and spc declined further by 2 log units and 1 log unit, respectively, at 21 days after which it was undetectable. CFU counts for all control strains and bioluminescent strains were 10^6 CFU/mL after challenge with the same concentration of BA (Figure 5.9B). A log unit reduction was observed for all strains up to 24 hours. Subsequently, a 2 log unit reduction was observed for all bioluminescent constructs and no CFU count was detected for negative control strains at 7 days. CFU count remained stable at approximately 10^3 CFU/mL for all bioluminescent strains between 7 and 14 days. Finally a log unit reduction in CFU count to approximately 10^2 CFU/mL was observed at 21 days and it then remained stable at 28 days. RFU readings followed the same pattern as CFU count for all strains tested (Figure 5.9C). Correlations between CFU count, bioluminescence and fluorescence were significant ($P<0.01$) with coefficients greater than 0.97. A 6 log unit reduction in CFU was observed at 7 days and > 2 log unit reduction in bioluminescence was observed at 28 days.

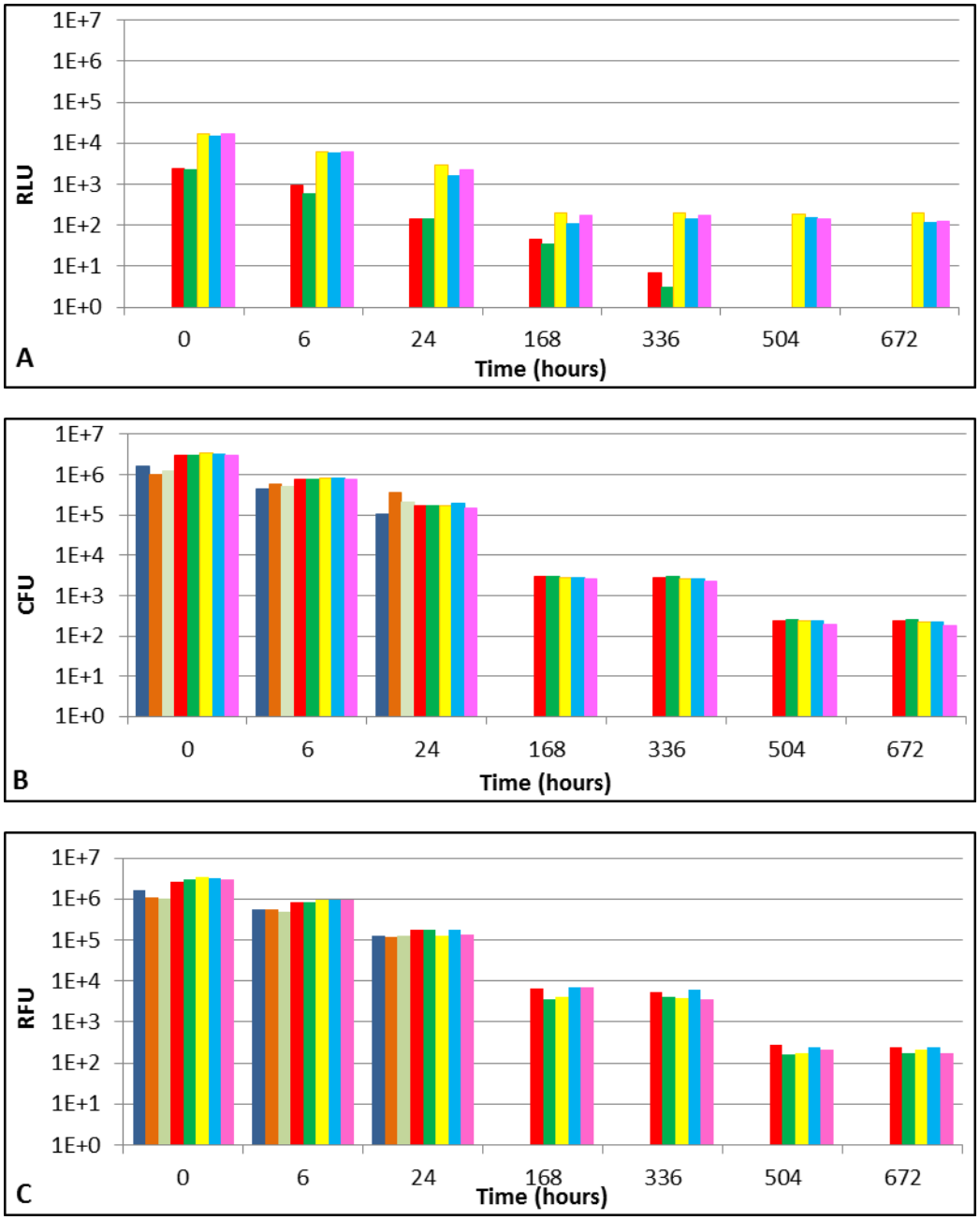


Figure 5.9: BA efficacy test for all strains challenged with 0.5% BA.
Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

WT pME4510 pME*lux* ldcC spc lysR25 lppR4 tatH5

Bioluminescence gradually decreased for all strains challenged with 0.25% BA (Figure 5.10A), by a half log unit within 24 hours. Subsequently bioluminescence remained stable for the duration of the studies. CFU count (Figure 5.10B) and RFU (Figure 5.10C) declined very slightly within 14 days, after which the CFU and RFU remained consistent at 10^5 CFU/mL and 10^5 RFU/mL, respectively, for all strains except the wild-type, which decreased further to 10^4 CFU/mL and 10^4 RFU/mL.

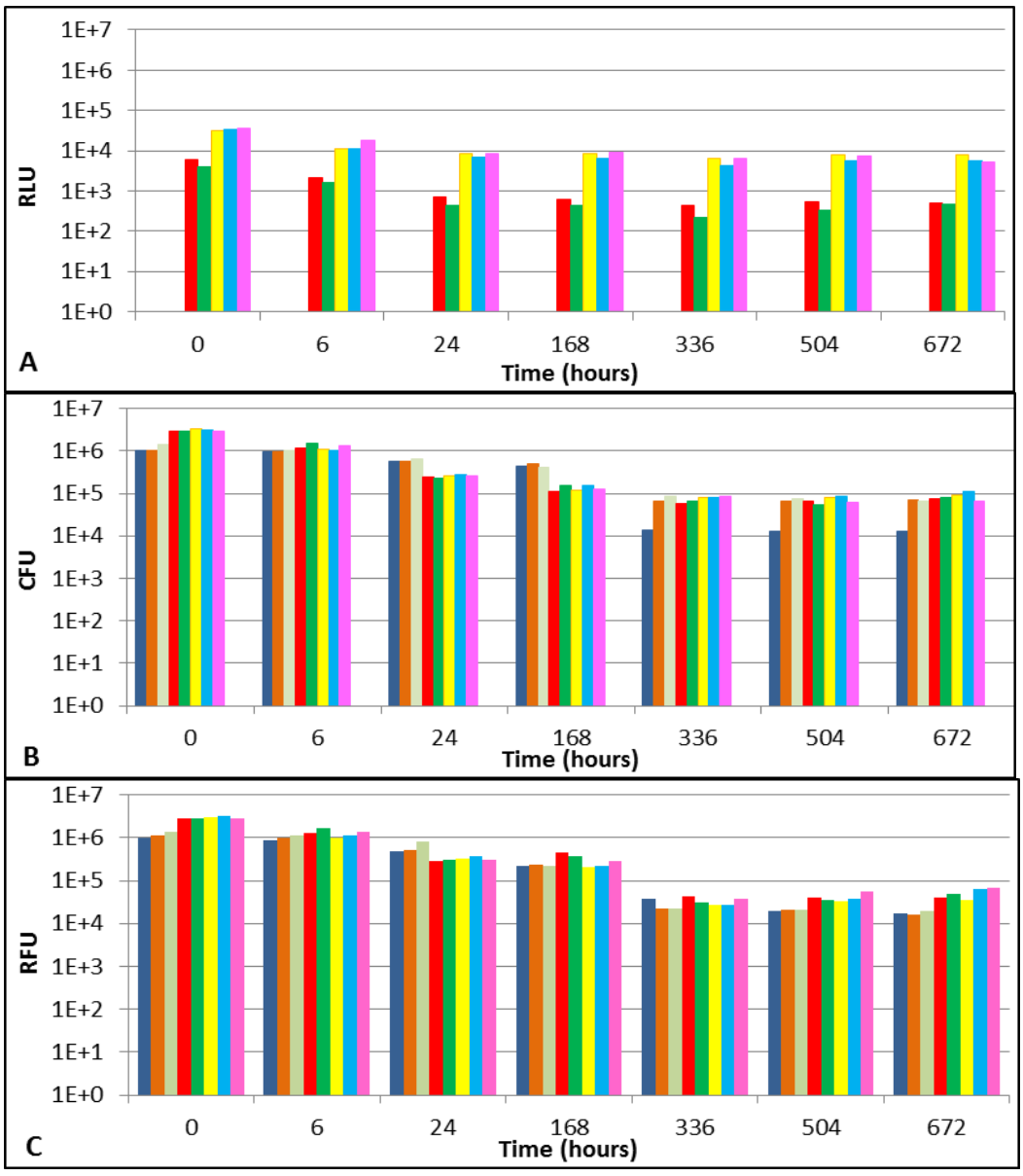


Figure 5.10: BA efficacy test for all strains challenged with 0.25% BA.
Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

WT pME4510 pME*lux* ldcC spc lysR25 lppR4 tatH5

A gradual reduction in bioluminescence was observed up to 24 hours, when all strains were challenged with 0.125% BA (Figure 5.11A). After 24 hours bioluminescence remained fairly consistent throughout the remainder of the experiments. CFU count remained consistent for the first 24 hours after which there was a log unit decrease for all strains (Figure 5.11B). Although CFU count of all the bioluminescent constructs remained consistent until the end of the experiment, the wild-type strain was found to increase very slowly at a rate of half a log unit within 3 weeks. Fluorescence data also showed a similar pattern (Figure 5.11C). Correlations between bioluminescence and CFU count were significant at $P<0.05$ (except for tatH5) with coefficients reduced to between 0.77 – 0.87 for all bioluminescent constructs. Correlations between bioluminescence and fluorescence were also found to reduce to a similar range with significance only at $P<0.05$. However, significant correlations between culturable cells and fluorescence were obtained, indicating that bioluminescence decreased before cell count decreased.

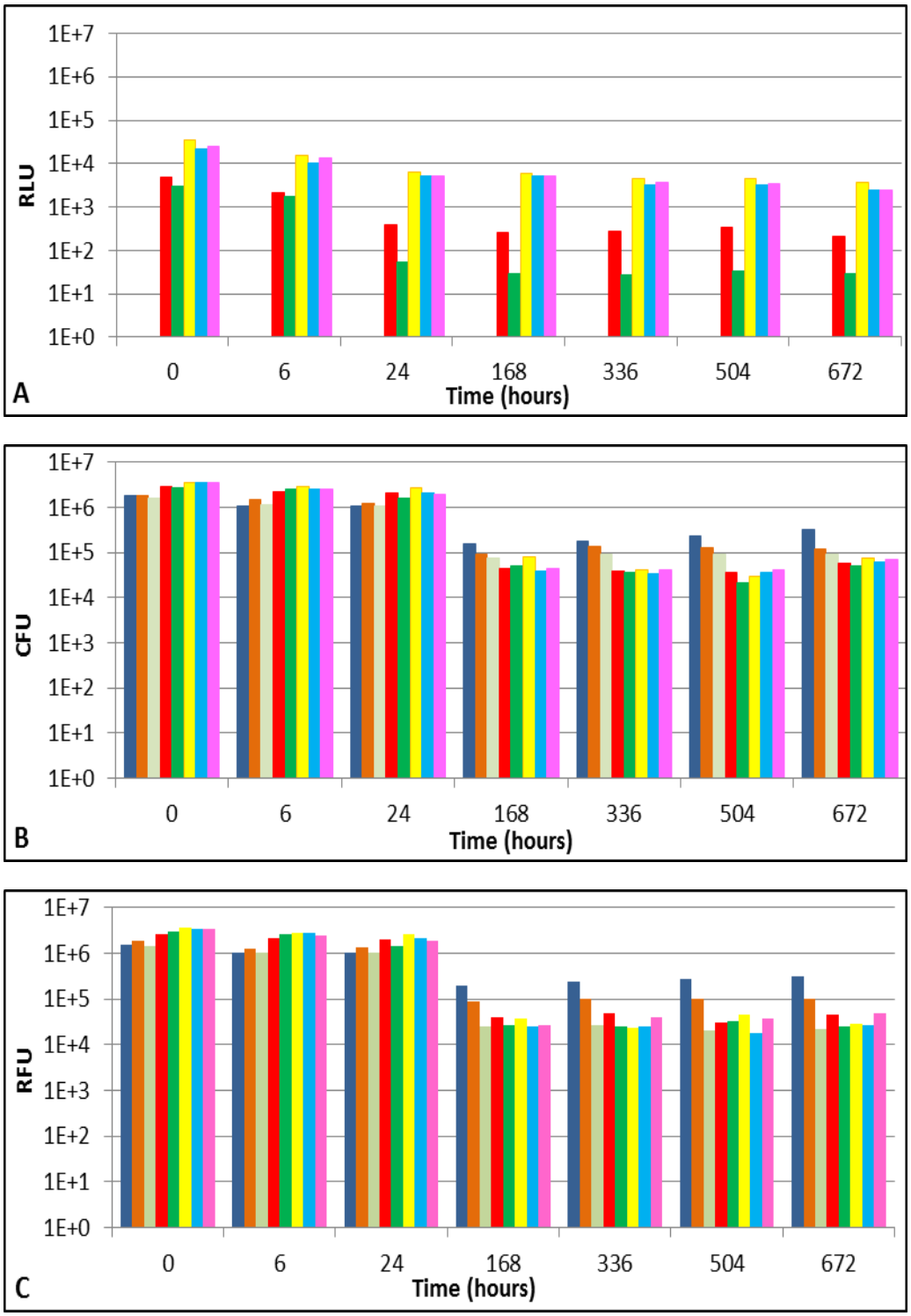


Figure 5.11: BA efficacy test for all strains challenged with 0.125% BA.
Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

Bioluminescence remained consistent throughout the duration of the experiment for all strains, when they were challenged with 0.06% BA (Figure 5.12A). No fluorescence experiments were performed for the strains challenged with 0.06% BA. Interestingly, CFU count increased by a half log unit within 28 days (Figure 5.12B). Correlations between bioluminescence and CFU count were significant ($P<0.01$) with r-values between 0.89 and 0.95 for all bioluminescent strains except spc where the correlation ($r = 0.76$) was significant at $P<0.05$.

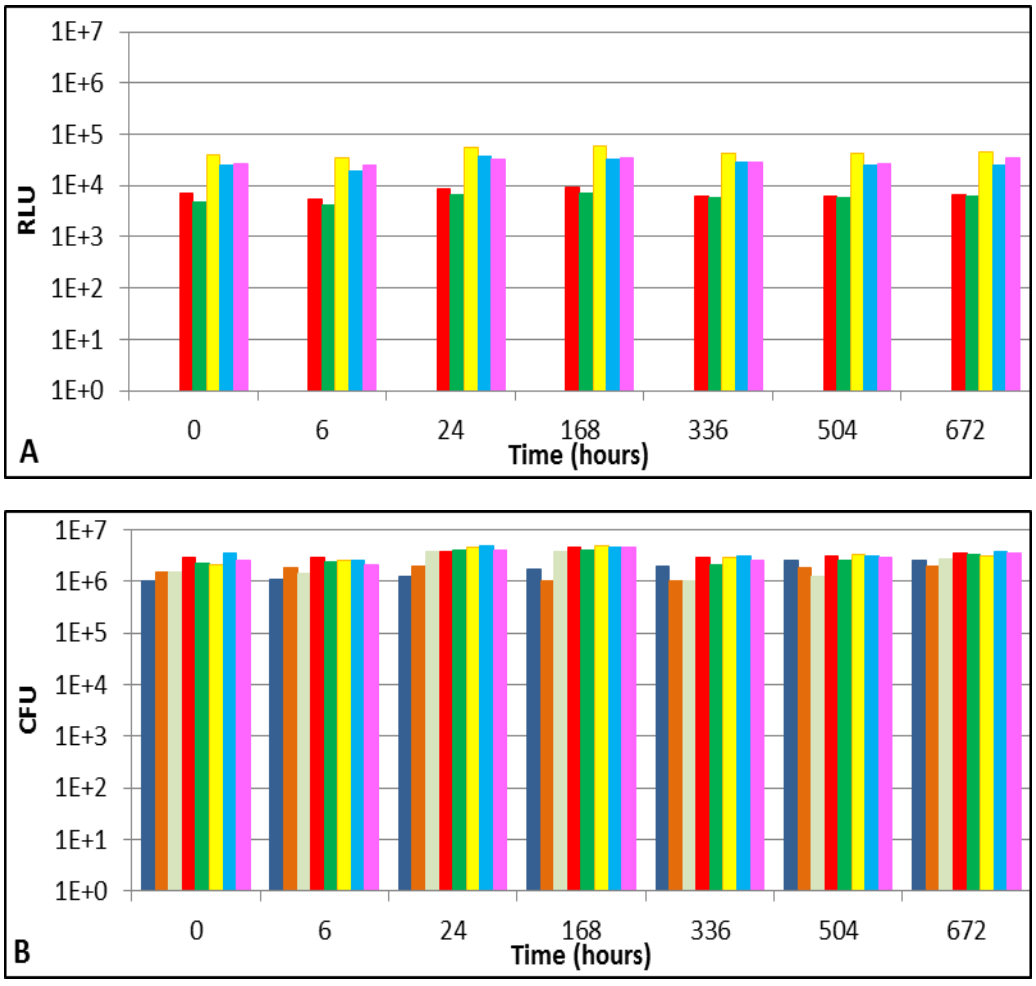


Figure 5.12: BA efficacy test for all strains challenged with 0.06% BA.
Measured at various time points up to 672 hours by (A) Relative bioluminescence, (B) CFU count and (C) Relative fluorescence.

WT pME4510 pME*lux* ldcC spc lysR25 lppR4 tatH5

Correlations between culturable cell numbers and fluorescence (>0.9) were significant (P<0.01) for negative control strains wild-type, pME4510 and pME*lux* when challenged with different concentrations of BA over 28 days.

5.5 Discussion

Benzalkonium chloride (BKC), a synthetic nitrogenous cationic molecule, is widely used as an antimicrobial preservative in food industries, hospitals and household products as well as pharmaceutical preparations (Fazlara & Ekhtelat, 2012). Its bactericidal properties have promoted its use in eyewashes, nasal sprays (Graf, 2001; Hodges et al., 1996; Lenoir et al., 2011; Marple et al., 2004) and ophthalmic solutions (Mohanty et al., 2012; Rase et al., 2011; Ryan et al., 2011).

Benzyl alcohol (BA) is a synthetic volatile aromatic alcohol that is widely used as an antimicrobial preservative in pharmaceutical preparations and cosmetic products (Scognamiglio et al., 2012). It is used increasingly in multi-dose protein and peptide products (Meyer et al., 2007; Roy et al., 2005). In this study, the efficacies of BKC and BA were assessed using the novel bioluminescent method in parallel with the traditional plate count method and fluorescence detection in an attempt to compare the three methods.

5.5.1 Minimum inhibitory concentrations of BKC and BA

All strains of *P. aeruginosa* were challenged with different concentrations of BKC, ranging from 0.0003% to 0.04%. The MIC for wild-type, pME4510 and pME*lux* was found to be 0.01%. This result has been confirmed by another study that found the MIC for wild type *P. aeruginosa* to be 0.01% (Osman, El-Hendawy, Hassan, Abdel-All, & Dina Ezzat, 2012). The MIC for bioluminescent constructs was found to be 0.00125%. Several studies have also shown that the MIC of BKC against *P. aeruginosa* is non-uniform. Studies found the MIC to be 0.006% (Fazlara & Ekhtelat, 2012) or 0.0032% (Kamysz & Turecka, 2005). Yet another study indicated the MIC to be 0.025% after 120 hours of contact time (Khajavi et al., 2007).

The change in MIC from 0.01% for wild-type to 0.00125% for bioluminescent constructs suggests that the bioluminescent constructs are more vulnerable to BKC challenge than the wild-type. The studies suggest that the incorporation of the *lux* cassette under constitutive promoters may be detrimental to the cells by providing a metabolic load on the cells. Furthermore, incorporation of just the *lux* cassette into the plasmid had no effect on the MIC. However, further incorporation of promoters changed the MIC significantly. The strength of the promoters, however, did not have any effect on the MIC shift. Physical observations of growth of bioluminescent constructs compared to that of wild-type also showed that bioluminescent constructs produced significantly less biofilms at 24 hours. This may explain the metabolic load/detrimental effects of active expression of the *lux* cassette that result in cells being more susceptible to benzalkonium choride. Gilbert and Moore (2005) have shown a positive correlation between biofilm formation and increase in MIC. Changes in MIC in gram-negative bacteria may be due to certain factors that alter the susceptibility of bacterial cells against preservatives. These factors include alterations in phenotype, efflux pumps, expression of enzymes, chromosomal and plasmid-based alterations (Gilbert & McBain, 2003). They also suggested that plasmids provide low levels of resistance against preservatives.

Strains were challenged with different concentrations of BA, ranging from 0.06% to 2%. The MIC for all strains tested using the bioluminescence and absorbance methods were 1%. Similar results have been shown in other studies (GE healthcare, 2012; Meyer et al., 2007). This shows that the bioluminescence method may be utilised instead of absorbance to determine MIC of antimicrobial agents. Such a method also provides a rapid frontline screening platform for testing novel antimicrobial agents for antimicrobial potential. Results from the MIC determination were used to determine the concentration range to be used for antimicrobial efficacy testing of BKC and BA.

5.5.2 Preservative efficacy tests for BKC and BA

Preservative efficacy testing (PET) was performed according to the requirements of the pharmacopoeias. This study aimed at utilising the novel bioluminescent strains to determine antimicrobial efficacy of BKC and BA using the bioluminescence method of detection in parallel to the traditional plate counting method. Different concentrations of BKC, ranging from 0.02% to 0.0003% ($^{w}/_{v}$) and BA, ranging from 0.06% to 2% ($^{v}/_{v}$), were used for the PET against all *P. aeruginosa* strains used in the study.

The MIC of BKC against the wild-type was 0.01%. However, under starved conditions in preservative efficacy testing, the wild-type strain was susceptible at 0.00125% BKC. Similarly, the MIC of BA for wild-type and bioluminescent constructs was 1%. However, it was surprising that significant bacterial growth and bioluminescence was observed at 24 hours for bioluminescent constructs challenged with 1% BA during the preservative efficacy testing. MIC determination is performed using growth medium, thereby providing cells with a chance to survive in the presence of antimicrobials by utilising the available energy sources. The change in biocide susceptibility relates to the fact that in nutrient-depleted environments (PET), the bacterial cells are slow-growing as opposed to nutrient-rich environments (MIC determination in presence of media) when the bacterial cells grow and form biofilms (Fazlara & Ekhtelat, 2012). In the presence of growth media, the cells were strong enough (unstarved) to provide intrinsic resistance against antimicrobials as compared to their susceptibility when the cells were starved during preservative efficacy testing. Biocide activity may be significantly compromised in nutrient-rich environments due to the biofilm formation in such environments. Nutrient depleted environments trigger a stress response by bacterial cells (Gilbert & McBain, 2003). Initiation of stationary phase leads to dormant phenotypes where cells adopt a ‘resting’ nature. A study (Foley, Marsh, Wellington, Smith & Brown, 1999) also showed increased rpoS expression in nutrient-rich environments. In nutrient limiting environments, the detrimental effect of metabolic burden has also been highlighted in a study

where lack of glucose has shown increased susceptibility of *P. aeruginosa* to benzalkonium chloride (Mc Cay, Ocampo-Sosa & Fleming, 2010). Lack of magnesium, which is vital for *Pseudomonas* cell membrane development, has also been shown to increase susceptibility of the cells to antimicrobial agents (Manzoor, Lambert, Griffiths, Gill & Fraise, 1999)

Observation of bacterial growth as evidenced by increase in bioluminescence and CFU count when cells were challenged with BA indicated that *Pseudomonas* cells utilise BA as a source of nutrients in the absence of growth media. A previous study has shown that *Pseudomonas aeruginosa* cells produce lipase that transesterify 0.2% benzyl alcohol to benzyl acetate (Singh et al., 2008). This phenomenon was also observed when *Pseudomonas* cells were challenged with 0.06% BA, where CFU count increased by a half log unit within 28 days.

Negative control strains were observed to be more vulnerable to BKC challenge than to bioluminescent constructs. This was indicated by the susceptibility of negative control strains at 0.0006% BKC, whereas the bioluminescent constructs were resistant to BKC at the same concentration. The promoterless strain also behaved in the same manner as the wild-type and hence it was more susceptible than the bioluminescent constructs. This indicated that the incorporation of the plasmid and therefore expression of the genes on the plasmid provided a protection against BKC for all bioluminescent cells. Growth of the promoterless strain in a manner similar to the wild-type suggested that it was not plasmid incorporation but rather the expression of the *lux* cassette that was conferring resistance of bioluminescent constructs to BKC.

Most interestingly, the correlation between bioluminescence and CFU count in the absence of preservative was weak and non-significant. However, the correlation between culturable cell count and fluorescence was significant ($P<0.05$), with a correlation coefficient greater than 0.8. This indicated that the cells were culturable. However, their bioluminescence pathway was affected. This was further confirmed with a

significant difference between the bioluminescent method and plate counting method when ANOVA was performed. Under starved conditions, bacterial cells lack the fundamental resources of energy production and therefore cannot perform certain energy-demanding reactions because of a lack of an energy source. One such example is the bioluminescence pathway, which is energy-demanding in the form of NADPH, $FMNH_2$ and ATP (Meighen, 1991). Other metabolic pathways include biofilm production and virulence determination.

High concentrations of BKC (0.02%-0.0025%) and BA (2%) were found to be too toxic for the cells, even though the inocula were 10^6 CFU/mL in accordance with the pharmacopoeial requirements. This was observed by immediate arrest in bioluminescence and significant reduction in CFU count, hence indicating an immediate bactericidal effect of the biocide. Typical in-use concentrations of BKC and BA as antimicrobial agents are 0.01-0.02% (Osman et al., 2012; Rase et al., 2011; Ryan et al., 2011) and 1-2% (GE healthcare, 2012; Meyer et al., 2007) respectively. The immediate bactericidal effect has been observed because cell membranes are virtually dissolved at such high concentrations of BKC and therefore immediate bactericidal effect is observed (Lambert & Hammond, 1973). At high BKC concentrations, micelles are formed leading to solubilisation of the hydrophobic membrane (Gilbert & Moore, 2005). BKC also forms dimers, which interact strongly with the cell membrane, thereby exerting a stronger antimicrobial effect (Gilbert & Altaae, 1985). A high concentration of BA is thought to cause protein denaturation and aggregation (Gupta & Kaisheva, 2003). Benzyl alcohol at a concentration of 1% causes soluble protein aggregates; thus the effect on bacterial cells is not as rapid as that of 2% BA (Gupta & Kaisheva, 2003).

Fluorescence detection with the aid of Molecular Probes® Live/Dead® BacLight™ staining comprising of two dyes Syto-9 and propidium iodide (Berney et al., 2007; Molecular Probes) discriminated between viable and non-viable live cells. These two dyes are DNA intercalators (Berney et al., 2007); Syto-9 crosses intact membranes of viable cells and fluoresces

green whereas propidium iodide is able to cross damaged membranes only (non-viable cells) and fluoresces red. Hence, viable cells and non-viable cells can be distinguished by their fluorescence output. This method of estimating viable and non-viable cells is a rapid method that has been used in numerous studies (Ferreira et al., 2011; Harrison et al., 2008). At BKC concentrations of 0.0006% and 0.0003% and a BA concentration of 1% correlations, between bioluminescence, CFU count and fluorescence were significant ($P<0.05$) for all bioluminescent constructs. However, a decrease in the correlation coefficients between fluorescence and bioluminescence and between fluorescence and culturability suggested that a few cells had become non-culturable. At low BKC concentrations, the membrane perturbations lead to loss of membrane fluidity and stable hexagonal arrangements of phospholipids are formed (Gilbert & Moore, 2005). Therefore cells may not be able to grow and replicate on media.

Although concentrations of 0.0006% BKC and 2% BA fulfilled the criteria of the pharmacopoeias, the latter does not take into account cells that are viable but undetected using the plate count method. This may further explain why microbial contamination has been a problem in numerous pharmaceutical products for several decades; a recent study by Tan *et al* (2013) found *P. aeruginosa* contamination in unopened toothpaste. Another recent study also showed the importance of detection of viable, non-culturable cells in the food industry (Elizaquível, Aznar & Sánchez, 2014). The capability of viable but non-culturable bacterial cells to cause product spoilage and potentially pose as a threat for infections has been shown in various studies (Colwell et al., 1996; Rahman, Shahamat, Chowdhury & Colwell, 1996). It is therefore imperative that testing protocols for preservative efficacy be improved such that all microorganisms bypassing the protocols are detected in due time. *P.aeruginosa* has been one of the many widespread opportunistic microbes to have been detected in various pharmaceutical preparations and cosmetics.

At mid-range BA concentrations of 0.5% and 0.25%, bioluminescence, CFU count and fluorescence were detected throughout 28 days and correlations between these three parameters were significant ($P<0.01$). Another study observed insignificant protein aggregation and precipitation at 0.5% BA (Gupta & Kaisheva, 2003), suggesting that bacterial cells would be viable.

At a lower BKC concentration of 0.0006%, correlations between bioluminescence and CFU count were significant ($P<0.05$) for all bioluminescent constructs Correlations between bioluminescence and fluorescence were reduced. Decrease in bioluminescence prior to cell count was observed when cells were challenged with 0.0006% BKC; bioluminescence decreased by two log units within 6 hours, whereas fluorescence reduced by a half log unit. CFU count also reduced by two log units, suggesting that bioluminescence decreased before cell viability decreased. Initial reduction in bioluminescence followed by reduction in CFU count indicates that the binding of the biocide to membrane proteins and creating osmotic gradients such that the constituents of the cells leak out (Lambert & Hammond, 1973) leading to subsequent disturbances to optimum environments for luciferase reactions. This is rapidly followed by reduction in CFU count where the cell constituents have leaked out to detrimental levels such that cell death is observed. Furthermore, denaturation of proteins and formation of protein aggregates (Gupta & Kaisheva, 2003) including the luciferase enzymes imposes an immediate detrimental effect on the bioluminescence pathway. Hence this phenomenon of reduction in gene expression prior to reduction in cell number may serve as an early indicator of the effects of preservative on microbial death and therefore the bioluminescent method may be used to detect widespread death of microbial cells during antimicrobial efficacy testing. Bioluminescence has been shown to give an earlier indicator of the health state of bacterial cells compared to traditional plate counting.

Correlations between bioluminescence, cell count and fluorescence measured at the minimum inhibitory concentrations of BKC were also

significant for all the bioluminescent constructs. Analysis of variance tests give an indication whether the bioluminescent and plate count method were equivalent. These tests were performed at $P<0.05$. For various concentrations of BKC and BA during preservative efficacy tests, it was found that there was no significant difference between the bioluminescent method and traditional plate counting method for concentration ranges of 0.0003% - 0.02% BKC and 0.25% - 2% BA as observed by P-values being greater than 0.05. The bioluminescent construct tatH5 had no significant difference between the bioluminescent method and traditional plate counting method at a concentration of 0.125% BA as well. This therefore indicated that the bioluminescent method may be used to detect microbes during antimicrobial efficacy testing. Previous studies have also utilised the bioluminescent method to examine the efficacy of biocides such as phenol, chlorhexidine digluconate and ethanol (Choi & Gu, 1999; Dhir & Dodd, 1995; Robinson et al., 2011). Testing model preservative systems used in pharmaceutical preparations against *P. aeruginosa* using the ATP bioluminescent method showed incompliance of the preservative systems to the pharmacopoeial requirements (Kramer et al., 2008). This was because initial ATP levels in *P. aeruginosa* were low (Kramer et al., 2008). ATP among other energy compounds are regarded as markers that inform about the global health state of bacterial cells. Therefore reduction in initial levels of such markers indicate immediate effect of antimicrobial agents on bacterial cells. Such immediate effects would not be detected by the ATP bioluminescent method since this method requires ATP extraction from cells, thereby presenting a physical disadvantage for detection of immediate effects. Coupling of bioluminescence to cell viability with respect to constitutive gene expression provides a powerful, non-destructive tool for studies of bacterial growth under preservative challenge in real time.

Bioluminescent signals as a result of gene expression from P_{ldcC} and P_{spc} were weak. As a result the strains with these promoters showed weaker and non-significant correlations between bioluminescence and CFU

counts when challenged with BKC concentrations between 0.00125% and 0.01%. There strains therefore are not suitable candidates for preservative efficacy testing.

Strong correlations between bioluminescence, CFU count and fluorescence were obtained for BKC concentrations between 0.0003% and 0.0025% against strains lys, lpp and tat. Similarly, strong correlations between the three parameters were obtained for BA concentrations between 0.125% and 2% against strains lysR25, lppR4 and tatH5. The bioluminescent method and traditional plate counting method were found to be equivalent for concentrations of 0.0003% - 0.02% BKC and 0.25% - 2% BA during preservative efficacy tests. The bioluminescent method and traditional plate counting method were found to be equivalent for construct tatH5 at a concentration of 0.125% BA as well. To conclude, these bioluminescent constructs are therefore strong candidates for selection for incorporation into a biosensor system for preservative efficacy testing.

6. General discussion and conclusion

In this study, bioluminescent constructs were developed from *P. aeruginosa* ATCC9027 using molecular cloning techniques. Insertion of the promoter-*lux* insert into the host plasmid pME4510 and subsequent transformation into *E. coli* DH5α cells was successfully achieved. *Pseudomonas* promoters were not well-recognised by the *E. coli* transcription machinery because the *Pseudomonas aeruginosa* bioluminescent constructs containing the same *P. aeruginosa* promoters produced bioluminescence of several magnitudes greater than that of their *E. coli* counterparts. A study by Rist & Kertesz (1998) also suggested that promoters originating from *P. aeruginosa* were not recognised by *E. coli*.

During the development of these bioluminescent constructs, it was found that plasmid pSB417 lacked SalI and PstI restriction sites. Construction of plasmid pSB417 by Winson *et al* (1998) included functional SalI and PstI restriction sites, amongst other restriction sites in the multiple cloning site. However, this study reports non-functional or lack of these two restriction sites in this plasmid as observed by failure to digest the plasmid with these functional restriction enzymes.

Transfer of plasmids from *E. coli* to *P. aeruginosa* was more challenging; however, optimisation of the electroporation buffer to suit *Pseudomonas* was vital in achieving successful results. Electroporation buffers consisting of salts aided in post-electroporation recovery of the cell membranes and therefore resulted in successful transformation. Other studies have also shown the importance of salts in transformation (Diver et al., 1990; Farinha & Kropinski, 1990). Inclusion of small amounts of salts such as magnesium chloride was beneficial to the recovery of *P. aeruginosa* cells after electroporation because it is important in the maintenance of structural integrity of the lipopolysaccharides (LPS) of bacterial membranes (Dennis & Sokol, 1995). All recombinant strains carrying promoter-*lux* inserts produced bioluminescence whereas the promoterless strain did not. Therefore novel bioluminescent constructs were successfully produced in *P. aeruginosa* ATCC 9027. These different constructs have been named:

P. aeruginosa ATCC 9027 + ldcC pME-*lux*

P. aeruginosa ATCC 9027 + spc pME-*lux*

P. aeruginosa ATCC 9027 + lys R25 pME-*lux*

P. aeruginosa ATCC 9027 + lys G25 pME-*lux*

P. aeruginosa ATCC 9027 + lpp R3 pME-*lux*

P. aeruginosa ATCC 9027 + lpp R4 pME-*lux*

P. aeruginosa ATCC 9027 + tat H5 pME-*lux*

P. aeruginosa ATCC 9027 + tat H9 pME-*lux*

P. aeruginosa ATCC 9027 + tat H14 pME-*lux*

Two other variants in *P. aeruginosa* ATCC 9027 were also produced. The promoterless variant bearing the *lux*-cassette on the plasmid was named *P. aeruginosa* ATCC 9027 + promoterless pME-*lux*. The other variant *P. aeruginosa* ATCC 9027 + pME4510 was also produced in *P. aeruginosa.* These may be used as a tool for further manipulation.

There is very limited information in the literature about promoter structure and characterisation in *Pseudomonas aeruginosa* and therefore the data from this study contributes to a better understanding of promoters from *Pseudomonas aeruginosa*. Promoter strength of the five selected promoters was investigated by determining the bioluminescence per cell count and further related to the respective promoter sequences. During the exponential phase of growth, the promoter strength decreased in the order of lpp>tat>lys>ldcC>spc while during the stationary phase of growth the promoter strength decreased in the order of tat>lpp>lys>ldcC>spc. Weak expression of LdcC was also shown by Lemonnier and Lane (1998). The importance of the tat pathway for the transport of proteins to the cytoplasmic membrane has been shown previously (Jack et al., 2001) and therefore high expression from P_{tat} would be expected. This has been shown because the promoter strength of P_{tat} was strong compared to the other promoters.

The importance of the spacer region was emphasized because P_{ldcC} and P_{spc} consist of spacer regions whose distances were 13bp and 19bp respectively. The consensus spacer region between -35 and -10 is 17 $\pm$ 2 bp (Mishra & Chatterji, 1993). Expression of genes from promoters with such spacer regions has been shown to be high compared to that of promoters with shorter or longer spacer regions (Stefano & Gralla, 1982). These two promoters were found to be the weakest of all five promoters investigated. P_{lpp} had the highest level of expression during the exponential phase compared to that of the other four promoters. P_{tat} had relatively stable expression during the stationary phase compared to that of the other promoters. P_{lpp} is also a binding site for rpoS18 (Yan et al., 2007), a transcritption binding factor that binds in response to stress during the stationary phase. Hence the expression of *lux* cassette from P_{lpp} was found to be altered during the stationary phase, such that its expression was less than that from P_{tat} and P_{lysS}. From the promoter strength and sequence data for the five promoters tested, it was indicated that the -10 region seemed to be crucial for constitutive gene expression in the exponential phase while the -35 region seemed to be crucial for constitutive gene expression during the stationary phase. This was confirmed by various other studies, which have shown that the upstream half of the -10 region is more important for recognition by σ factors (Dombroski, 1997; Dombroski, Johnson, Lonetto & Gross, 1996; Dombroski, Walter, Record, Siegele & Gross, 1992) and subsequently for successful gene expression.

Bioluminescent constructs were validated successfully in accordance with various regulatory documents (ISO, 1994a, 1994b, 1994c; PDA, 2000), using the bioluminescent method. Bioluminescence was found to increase proportionately to CFU count for the range between 10^4 and 10^8 CFU/mL for strong promoters and between 10^5 and 10^8 CFU/mL for weak promoters. The lower limit of detection was estimated to be 10^2 RLU, as observed by the bioluminescence detection from bioluminescent constructs as compared to negative control strains. The sensitivity of the bioluminescent method was 10^3 CFU/mL. According to a review of

methods for rapid microbiological assays (Shintani et al., 2011), the sensitivity of the ATP bioluminescent method was greater than 10^2-10^3 CFU/mL and therefore the sensitivity achieved in this work was comparable to that of the ATP bioluminescent method. It was observed that bioluminescence of undiluted cultures (10^8 CFU/mL) was less than that of 10-fold diluted cultures, implying that turbid undiluted cultures hindered the accurate detection of bioluminescence by diffracting light away from the detector. This is known as the inner-filter effect (Kao et al., 1998). However, the linearity validation indicated that the requirement of 10^6 cells of inoculum for preservative efficacy testing was within the linear range and therefore the inner filter effect would not affect preservative testing regimes.

The percentage recovery of cells indicated the level of accuracy of the bioluminescent method compared to the traditional plate count method and should lie between 70% and 130% (PDA, 2000). Percentage recoveries using the bioluminescent method were between 70% and 130% for all bioluminescent strains; therefore, the bioluminescent method was accurate. Precision of the bioluminescent method was measured by relative standard deviation (RSD) of 10 replicates and should be less than 30% (PDA, 2000). Furthermore, statistical analysis by F-test and one-way analysis of variance showed no significant difference in precision between the bioluminescent method and the plate counting method for all bioluminescent strains. Therefore, the bioluminescent method passed the criteria set out in various documents for a microbial testing method (ISO, 1994a, 1994b, 1994c; PDA, 2000). Precision testing using the bioluminescent method for all constructs resulted in a RSD less than 15% for the range 10^4 to10^8 CFU/mL. The ATP bioluminescent method, which is accepted as an alternative PET method according to the pharmacopoeia, resulted in RSDs of 24.9% and 26% for 10^8 and 10^4 CFU/mL, respectively (Kramer et al., 2008), whereas the precision of bioluminescence for the constructs designed herein was less than 10% and less than 15% for 10^8 and 10^4 CFU/mL, respectively. This indicated

that the whole cell microbial bioluminescent method was more precise than the ATP bioluminescent method.

The bioluminescent method was also deemed equivalent, accurate and precise in the range 10^4 to10^8 CFU/mL under the criteria for determining these parameters (ISO, 1994a, 1994b, 1994c, 2000; PDA, 2000). Correlations between the bioluminescent method and plate count method were strong and significant when the strains were tested for accuracy, precision and linearity. No significant difference between the bioluminescent and the plate counting method was also found in other studies (Quilliam et al., 2012; Sun et al., 2012; Thorn et al., 2007). Equivalence measurements by analysis of variance showed no significant difference between the bioluminescent and plate count method. Therefore, the bioluminescent constructs gave equivalent or better accuracy, precision and linearity compared to the plate count method and met the validation criteria. All the recombinant constructs were validated successfully. This means that the bioluminescent constructs have a great potential for use in PET. Although numerous studies have suggested replacement of the traditional plate counting methods with whole cell microbial bioluminescent methods (Willardson et al., 1998; Wise & Kuske, 2000), it is believed that this study reports for the first time the use of validated bioluminescent constructs developed in a pharmacopoeial-defined test microorganism *P. aeruginosa* ATCC9027 in microbiological testing methods.

Stability of plasmids was regarded as vital for this study, since preservative efficacy tests require that stable plasmids are maintained throughout the duration of the test. All plasmid copy numbers remained constant throughout the 28 day period, indicating that the plasmids were stably maintained by the cells. Plasmid copy number for all strains was greatest during the exponential phase of growth, where pME4510 strain had 120 copies and the recombinant constructs had 100 copies. This is the phase when the cells are dividing rapidly and therefore the plasmids are replicating before the cells replicate. During the stationary phase of

growth, plasmid copy number was found to be approximately 80 and 50 for strain pME4510 and recombinant constructs respectively. Previous studies have also shown that plasmid copy number decreases as plasmid size increases (Smith & Bidochka, 1998).

The growth and death kinetics for all strains developed were compared to that of the wild-type and the performance of the bioluminescent method was assessed for determination of the health states of all constructs at various growth phases. Good correlations were observed between bioluminescence and fluorescence for the 28 day period for all bioluminescent constructs indicating that all viable cells were bioluminescent. The lag phase for all bioluminescent constructs lasted for approximately 3 hours, as indicated by peaking on the RLU:CFU plot indicating increased gene expression per bacterial cell count, which may have been due to an increase in plasmid copy number. Increased gene expression during lag phase was also defined by Al-Qadiri *et al* (2008). This aspect of bioluminescence detection prior to cell replication may provide a useful tool in early microbial growth detection. Following the lag phase, all strains entered the exponential growth phase for up to approximately 12 hours. Tailing of the peaks on the RLU:CFU plot was observed as the gene expression per bacterial cell count stabilised to linear relationships. Constant gene expression during the exponential phase of growth was also shown by Al-Qadiri *et al* (2008) and Thorn *et al* (2007). Bioluminescence has therefore been shown to give an indicator of the health state of bacterial cells comparable to that of traditional plate counting. The onset of the stationary phase was observed at 12 hours, after which there was no net increase or decrease in bioluminescence or bacterial cell counts. From the RLU:CFU plot, it was observed that constant light was produced per bacterial cell after 12 hours up to 48 hours for strong promoters. After 48 hours of growth, bioluminescence production was not proportional to bacterial cell count. A decrease in bioluminescence from P_{ldcC} and P_{spc} during the stationary phase may be

due to these promoters being less recognised by sigma-38, which is predominant during stationary phase (Raivio & Silhavy, 2001).

A metabolic load of gene expression of the *lux* cassette was observed on bacterial cells. The luciferase pathway is highly energy-dependent and utilises myristol-ACP as the lipid substrate (Close et al., 2012), which is also essential for membrane lipid formation. Hence, there may be competition for myristol-ACP between membrane lipid formation and the luciferase pathway. Myristol-ACP may be utilised in the luciferase pathway at the expense of membrane formation, thereby reducing the rate of formation of cell membrane of bioluminescent constructs compared to that of wild-type. This hypothesis of metabolic load was supported by various observations. Firstly, growth of the bioluminescent strains was slower than that of the promoterless strain, which grew in a similar manner to the wild-type. Secondly, the change in MIC from 0.01% for wild-type and pME*lux* to 0.00125% for bioluminescent constructs suggested that the bioluminescent constructs were more vulnerable to BKC challenge compared to the wild-type. Incorporation of the *lux* cassette under constitutive promoters was detrimental to the cells by providing a metabolic load on the cells. Thirdly, physical observation of bioluminescent cell growth compared to wild-type also showed that bioluminescent constructs produced considerably less biofilm at 24 hours than the wild-type. Hence, this may be the cause of the increased susceptibility to BKC against bioluminescent constructs as opposed to the wild-type, which explained the metabolic load/detrimental effect of expression of the *lux* cassette on bioluminescent constructs as compared to wild-type *P. aeruginosa*. A study by Gilbert and Moore (2005) has also shown a positive correlation between biofilm formation and increase in MIC. Therefore, decrease in biofilm formation would increase susceptibility of bioluminescent constructs to BKC.

A decrease in culturability of bioluminescent constructs by comparison with wild-type may suggest that the metabolic load on the bioluminescent constructs played a crucial role in converting cells towards VBNC states

when conditions were stressful, since bioluminescent constructs did not have the ability to replicate. Weaker correlations between cell culturability and fluorescence were observed during growth and death phases as a result of differences between these two variables. Wild-type cells were culturable throughout 28 days; however, the culturability of the promoterless strain and bioluminescent strains decreased at 7 days and 14 days, respectively, indicating that not all cells were culturable. Therefore, conversion of phase from stationary phase to long-term stationary phase seems to occur between 7 and 14 days. However, the correlations between bioluminescence and fluorescence using vital staining indicate that only viable cells are expressing the *lux* cassette. A decrease in relative bioluminescence also suggested reduced gene expression; another characteristic of possible VBNC state (Navarro Llorens et al., 2010). Viable but non-culturable states were also observed under preservative challenge. This was supported by a decrease in bioluminescence by two log units within 6 hours, whereas the fluorescence was decreased by a half log unit when all bioluminescent constructs were challenged with 0.0006% BKC.

In this study, the use of bioluminescent constructs as biosensors was evaluated during antimicrobial efficacy testing using BKC and BA. The study has shown that bioluminescence is suitable for monitoring health states of *P. aeruginosa* constructs. The bioluminescent method was able to measure different levels of microbial population (CFU) in media as well as under preservative challenge. Strong correlations between bioluminescence and plate count were statistically significant during growth and death kinetic studies. Furthermore, it was possible with the bioluminescent method to study the effects of preservative on the viability of bacterial cells. During BKC and BA PET, good correlations between bioluminescence, cell count and fluorescence were obtained for all the bioluminescent constructs. These correlations were also statistically significant, therefore indicating that the bioluminescent method may be used to detect microbes during antimicrobial efficacy testing. Coupling of

bioluminescence to bacterial metabolism with respect to constitutive gene expression provides a powerful, non-destructive tool for studies of bacterial growth under preservative challenge in real time.

At concentrations of 0.0006% BKC and 2% BA, 2 log unit and 3 log unit reductions in CFU at 6 hours and 24 hours for all bioluminescent constructs indicated that these concentrations of BKC and BA fulfilled the pharmacopoeial requirements of a preservative challenge (European Pharmacopoeia, 2008). However, the presence of non-viable cells may further explain why microbial contamination has been a problem in numerous pharmaceutical products for several decades; the recent study by Tan *et al* (2013) found *P. aeruginosa* contamination in unopened toothpaste. Another recent study also showed the importance of detection of viable but non-culturable cells in food industry testing (Elizaquível et al., 2014). It is therefore imperative that testing protocols for preservative efficacy are improved, such that all micro-organisms are detected in due time. *P. aeruginosa* has been one of the many opportunistic microbes to have been detected in various pharmaceutical preparations and cosmetics. Testing model preservative systems used in pharmaceutical preparations against *P. aeruginosa* using the ATP bioluminescent method showed incompliance of the preservative systems to the pharmacopoeial requirements (Kramer et al., 2008). This was because *P. aeruginosa* had reduced initial ATP level (Kramer et al., 2008). ATP, among other energy compounds is regarded as a marker that informs about the overall health state of bacterial cells. Therefore, reduction in initial levels of such markers indicates an immediate effect of antimicrobial agents on bacterial cells. Such immediate effects would not be detected by the ATP bioluminescent method, since this method requires ATP extraction from cells, thereby presenting a physical disadvantage for detection of immediate effects. For the strains with strong promoters, strong correlations between bioluminescence, fluorescence and culturability indicate that a large number of cells were viable, culturable and expressing the *lux* cassette. Coupling of bioluminescence to bacterial

metabolism with respect to constitutive gene expression provides a rapid, powerful and non-destructive tool for studies of bacterial growth during various growth phases in real time.

The relationship between antimicrobial concentration and response of microbes was good, as indicated for all concentrations and strains during MIC determination. The efficacies of BKC and BA were assessed using the novel bioluminescent method in parallel with the traditional plate count method in an attempt to compare the two methods. The MICs for BA against all strains tested were found to be 1% using bioluminescence and absorbance methods. This showed that the bioluminescence method may be utilised instead of the absorbance method to determine the MIC of antimicrobial agents. Such a method also provides a rapid frontline screening platform for testing novel antimicrobial agents for antimicrobial potential.

It was concluded that strains with P_{ldcC} and P_{spc} were not suitable candidates for preservative efficacy testing because of the decrease in bioluminescence during the stationary phases of ldcC and spc strains and because both strains produced weaker bioluminescent signals than other bioluminescent constructs. Good correlations between bioluminescence, CFU count and fluorescence were obtained for BKC concentrations between 0.0003% and 0.0025% against strains lys, lpp and tat. Similarly, good correlations between the three parameters were obtained for BA concentrations between 0.125% and 2% against strains lysR25, lppR4 and tatH5. There was no significant difference between the bioluminescent method and the traditional plate counting method for concentrations ranging from 0.0003 - 0.02% BKC and 0.25 - 2% BA. The bioluminescent method and traditional plate counting method were equivalent for concentrations of BKC (0.0003 - 0.02%) and BA (0.25 - 2%) during preservative efficacy tests. These bioluminescent constructs therefore are good candidates for selection for preservative efficacy testing. At greater concentrations, the bioluminescent method and traditional plate counting method were also found to be equivalent for

construct tatH5 at a concentration of 0.125% BA. Together with the results from growth and death kinetics, where tatH5 showed the greatest constitutive expression, it can be concluded that this candidate is preferred for testing various antimicrobial agents.

Preservative efficacy testing using traditional methods takes at least 28 days plus set up time and incubation time; additionally it is very laborious and time-consuming and is usually repeated. Furthermore, a range of concentrations of various preservatives need to be tested for antimicrobial efficacy, making the challenge test expensive, prolonged and laborious. Therefore, industries are reluctant to invest their finance, knowledge and equipment to conduct these tests. Use of whole cell microbial bioluminescent constructs therefore provides a rapid, accurate, non-destructive and less laborious method that gives results in real time. Results from validation of the bioluminescent method using *P. aeruginosa* bioluminescent constructs were accurate and precise across a linear range of microbial population. The whole cell microbial bioluminescent method has proved to be equivalent/better than both the traditional plate counting method and the ATP bioluminescent method. Therefore, the whole cell microbial bioluminescent method provides a platform for rapid antimicrobial efficacy testing. Furthermore, the bioluminescent method could be applied as a rapid, front-line screening platform for determination of minimum inhibitory concentration of novel compounds in pharmaceutical, food and cosmetics industries.

Coupling of bioluminescence to cell viability with respect to constitutive gene expression provides a powerful, non-destructive tool for studies of bacterial growth under preservative challenge in real time. Bioluminescence provides an earlier indicator of the health state of bacterial cells than traditional plate counting. By validation, growth and death kinetics, and subsequent antimicrobial efficacy testing, it has been shown that the bioluminescent method has several advantages over the traditional plate count method. Firstly, results are produced in real time. Secondly, use of the bioluminescent method allows early detection of

microbial cell death during death kinetics or in response to challenge with antimicrobial agents since they express the *lux* cassette constitutively, these bioluminescent constructs have the ability to screen a broad spectrum of antimicrobial agents. Due to high risk of contamination during sampling of cultures over a 28 day period, the bioluminescent method reduces the dectection of contaminating microbes that would normally be detected as positives with the plate counting method. Hence, the bioluminescent method reduces the detection of false positives and thereby provides specificity. Cell viability can be monitored in the presence of various concentrations of preservatives with this method, as opposed to the detection of cell culturability using the plate count method.

In conclusion, this study reports for the first time the construction and development of functional whole cell microbial bioluminescent constructs in *Pseudomonas aeruginosa ATCC 9027* using constitutive promoters for examination of overall health states of *Pseudomonas* cells when challenged with various antimicrobial agents. From the results and experience obtained from this study, it can be concluded that the bioluminescent method represents a valid alternative method for preservative efficacy testing in pharmaceutical development. It is an economical method with respect to labour, time and materials required. The bioluminescent method of detection provides a rapid, simple, sensitive, quantitative and cost-effective way of detecting microbial content in real-time without the need for laborious and destructive extraction procedures and expensive reagents.

Future work will include conducting reproducibility studies as part of the validation of the bioluminescent constructs. Such a validation should include precision testing in different laboratories, different analysts, different reagent batches and different instruments but using the same analytical protocol.

Following a full validation, pharmaceutical formulations should be tested against the bioluminescent constructs for testing antimicrobial efficacy

using the bioluminescent method. Currently, the preservative efficacy testings were performed with preservatives in water. According to the pharmacopoeias, full pharmaceutical formulations, such as ophthalmic solutions, should be tested to assess any interfering components of the formulations that may stimulate or hinder the growth of micro organisms.

The bioluminescent constructs should be tested as part of consumable products that would be supplied to consumers. The bioluminescent constructs are to be supplied as freeze-dried strains in sterile multi-well plates. The multi-well plates may contain different concentration of micro organisms with positive and negative controls. The plates may also contain different micro organisms that are specified in the pharmacopoeia. The plates would be placed in an incubated luminometer connected to a signal processor that will automatically process data in real time mode. Such a system would therefore form a functional biosensor system.

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Appendices

Appendix 1: Promoter sequences

LdcC sequence

Locus: NC_002516 2256 bp DNA linear
Definition: **Orn/Arg/Lys decarboxylase LdcC**.
>gi|110645304:1974821-1977076

CCTGCCCATGCACCACGGGCGCCGCGTTCAGCGTGTCGCCCGGTCCACCAGCAAGGTGTCGCAGAACTGCTCG
AAACGGCGGATCCTGCCGTCCTCTACCGTCCATACGTGTGCCACACGTGCTTCGAACGACTTGCCGCTGCGAC
GGTGGGTCGCTGCATATCGACCGACGGCGATCACGCTGTCGCCGCCGTCGATCAGGCGCTCGATCTCGACCCT
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GCCAGCGGGAAACCGGCCATTTCCGTCCATTCGGTGCGTTCGTCCATGTCCGCCAGCATTCCGGCGAGGTCCC
CACGGCTAGAGGCCGCATAGTGATCGCTGACGATCCGATAAGCGCTGCGCATCGTTGTCGCTCCGTAGCAGGG
CAGAGAGGTCCATGCTCCTCGCTGGCGGGAGCGGAGGCAAGCGCGGCGCGCCGACTTGTGTTGCCGCGACCTT
TTCTTACAAGGGCAAGCCGCCGGGGGTTACGCGCGTGCCGGCAAGTCTCTACCCTGAGCCGACCGGCGGGGTT
CGCCCGCCTGCTGCTCGGGATCAACAATGTATAAAGACCTCAAATTTCCCGTCCTCATCGTCCATCGCGACAT
CAAGGCCGACACCGTTGCCGGCGAACGCGTGCGGGGCATCGCCCACGAACTGGAGCAGGACGGCTTCAGCATT
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CAACTATTCCTGGCATACGCCGGGCCACGGCGGCGGTGTCGCCTATCGCAAGAGTCCGGTGGGACAGGCGTTC
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GATCGCTCGGTTGGAAGGGCGCATCGCGGCCGTCATGCTGGTACCCTATCCGCCGGGTATCCCGCTGATCATG
CCGGGTGAGCGCTTCACCGAGGCGACCCGCTCGATCCTCGACTATCTCGAGTTCGCGCGGACCTTCGAGCGCG
CCTTCCCTGGTTTCGACTCCGATGTGCATGGCCTGCAGCATCAGGACGGACCGTCCGGGCGCTGCTATACCGT
TGAATGCATAAAGGAATGA

hypothetical protein ldcC

Forward Primer 5’-CTTCAAGAATTCGGGTTACGCGCGTGCCGG-3’
Reverse primer: 5'-CTGATTACGTAGTCAAGAGAATGCTGAAGCCGTCCTG -3'

Softberry analysis
Promoter Pos: 1878 LDF- 0.47
-10 box at pos. 1863 ATGTATAAA Score 44 (part of gene start position also)
-35 box at pos. 1844 CTGCTG Score 12

LolB sequence

LOCUS: NC_002516 618 bp DNA linear
DEFINITION: Outer membrane lipoprotein lolB.
>gi|110645304:5236774-5237391

CACGCCTTGCTCGAACAGGTGGCGCGCGACCAGGGTGATGGTCTCGCCGGCGCCGATCAGCAGCGCCTGGCTG
CGGTGCAGGTCGCTGAAGATCTGCTTGGCCAGGCTCACCGCGGCGAACGCCACGGACACCGGGTTCTCGCCGA
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TATGTGGTGCAGGCGCAGAAAACCCGCGACCCCGGGGTTTCCGAGCGCGCCTTCCGCATCGCCGAATACCTCG
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CTGGCGCAGATGGAAAAGGACCTGCGCTTCGTCATCGCCCGCGAGCCGGACAACGCCATGGCCCTCAACGCCC
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CCTGGCGCGCCTGGAACAGGACGGCTGGCAGATCGAATACACCCGCTACGCCGAACAGAACGGCTACTGGCTA
CCCGAGCGCCTGAAGCTGCACGGCCAGGACCTCGACGTCACCCTGGTGATCAAGGACTGGCAGCCGCGCCAGC
TCGGCCGCTGA

HemA Hypothetical protein lolB

Forward primer 5’-CTTCAAGAATTCGTATTGACCCCATAGACAGCTTCG-3’
Reverse primer: 5'-GTGATTACGTAGTCAGCGCAAGGGATTTGTTCATA-3'

Softberry analysis
Promoter Pos: 235 LDF- 1.61
-10 box at pos. 220 TGTCATGAT Score 51
-35 box at pos. 200 TGGGCT Score -4

LysS sequence

LOCUS AE004091 1506 bp DNA
DEFINITION Lysyl-tRNA synthetase
>gi|110645304:4140884-4142389

GCTCGGCCAGCAGGTGCTCGCGGCGCTCGGTGAGCTGGACCTGGACATACTGCACGGCAAGCTCGACGATCCG
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GGCCCGTACGCCGTCGCGGACGATCAGGCGCATGGCGGCATCGAGAATCGCCTGGCGCCGCTGCTCGCTGCCC
TGGCGGCTGGCCTTGCGGCCCTGGTAACGAACGCTTTCGGCTACCGCGCTGGCGGCCTGCGCGGCGCTGTCTC
TGGGAGCTACTCGGGACACGGTGTTTCCTCCGCTCGCGGACGATGGGGCATTGTTCGGACTTTTTCGCGTGGA
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GCCTGGTCCTCGGCGTCGTTCAGCTCGGAATAGGCATTGGCGATCTCGCGGCCGCCGATGAACAGCTCGAAGC
GGTCGGTGACGCTCGGGTCTTCGTCGTTGCGGCGGGCCAGCGGCGAAACCTCGAAGGGGTAGCGGGTGATGAA
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CCGAGGACCTTGGCGCCGGCCTTCTTGGCGATGGCCCGCGCCTTCTCGACGTCGTTGAGGTCGGCGGCGGTGA
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CGCCTTCGTTACGGAAGTTGCGGTTGATCTCGAAGACCTTCTCGAAACCGCCGACCACCAGGCGCTTGAGGTA
CAGCTCCGGGGCGATGCGCAGGAACATGGCCATGTCCAGCGCGTTGTGATGGGTCTCGAACGGCTTGGCCGCG
GCGCCGCCGGGGATGGTCTGCAGCATCGGGGTCTCCACTTCGAGGAAACCGCGCTCGCTGAGGAAACGGCGGA
TGTGGGCGATGACCTGGGAACGCACGCGGAAGGTGTGGCGGGTTTCCTCGTTGACCATCAGGTCGACGTAGCG
CTGGCGGTAGCGCTGCTCGGTGTCGGTCAGGCCATGGTGCTTGTCCGGCAGCGGACGCAGCGACTTGGTCAGC
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GGGATCGCTGCGGCTTCCAGCTCTTCCTTGGTCTTGTCGGCGTACTGTTTCTGCAGGTCCGCGAAGTAGGCGT
CGCGGCGGAAGTCGTTGGGGAAGGCGATGGCCCGGGCTTCGCGCACGGCGGCAAGTTTTTCCTTGCGCTGGGC
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Forward primer: 5'-CTTCAAGAATTCGCTGTCTCTGGGAGCTACTCG-3'
Reverse primer: 5'-GTGATTACGTAGTCAGATAGCGGCCGATTGATTC-3'

Softberry analysis

Promoter Pos: 831 LDF- 0.47
-10 box at pos. 816 GGGCATTGT Score 27
-35 box at pos. 794 TTTCCT Score 37

Spc sequence

LOCUS AE004091 1506 bp DNA
DEFINITION spc ribosomal protein operon
>gi|110227054: 4756935 to 4760468.

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CAGCCTTCACGGATCTTGAAGCCTGCGATGGACTTACGAGCGTAAGTCACGACCGGCTTCTGACCAGTGATCT
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CGGATGATGGAACCGTCGGTACGACGCACGCCGTGCTTGGTGCGAACGACCACGGCAGTCATGACCTGGCCCT
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GGTCCAGGCCTTGGTCTTGGCCAGCGGACGGGTTTCGCGAATGGTGACCAGGTCACCAATGCGGCACTGATTG
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rplE rplX rplN rpsQ

Forward primer: 5’-CTTCAAGAATTCGTAACGCGCGGAACTTCCATC-3'
Reverse primer: 5'- CTCATTACGTAGTCAAGGTAGATCACCATGGCACGA -3'

Softberry analysis
Promoter Pos: 4976 LDF- 1.33
-10 box at pos. 4961 CGATAAATT Score 51
-35 box at pos. 4936 TTTCAG Score 30

Tat sequence

LOCUS NC_002516 1488 bp DNA linear
DEFINITION Twin arginine translocase protein
>gi|110645304:5706552-5708039

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GGCTGGTCGCCCTGCTGGTGCTCGGCCCCGAGCGCCTGCCGGGCGCCGCACGTACCGCCGGCCTGTGGATCGG
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TGGTCCTGACCCCGCCGGACATCTTCTCCCAGACCATGCTCGCGGTGCCGATGTGGCTGCTGTTCGAGATCGG
CCTGCTGTTCGGCCGCCTGGTACGCAAGCGCGGCGAGCATCCGGACGACCAACCGGCCAGCGACGGCGACCAG
CCTCCGGCCACCCGCCAGTGA

UbiB HisI HisE tatA tatB tatC

Forward primer 5’-CTTCAAGAATTCGGGGTATTCCTGATCCTGCGCCG-3'

Reverse primer: 5'-GTGCATACGTAGTCAGGCGTTCCAGTGAATCTCATCCAG-3'

Softberry analysis

Promoter Pos: 2508 LDF- 1.09
-10 box at pos. 2493 TGGCACACT Score 42
-35 box at pos. 2473 TAGCCA Score 24

Appendix 2: Promoter characterisation

Table 7.1: Characterization of each of the genes (Price, Huang, Alm, & Arkin, 2005).

Accession number	Start position	Stop position	Strand	Systematic name	Gene name	NCBI Description
NP_250037.1	1461854	1460307	-	PA1346		Arginine/lysine/ornithine decarboxylases
NP_252390.1	4142389	4140884	-	PA3700	lysS	Lysyl-tRNA synthetase
NP_252927.1	4754379	4753990	-	PA4237	rplQ	50S ribosomal protein L17
NP_252928.1	4755424	4754423	-	PA4238	rpoA	DNA-directed RNA polymerase, alpha subunit/40 kD subunit
NP_252929.1	4756067	4755447	-	PA4239	rpsD	30S ribosomal protein S4
NP_252930.1	4756473	4756084	-	PA4240	rpsK	30S ribosomal protein S11
NP_252931.1	4756848	4756492	-	PA4241	rpsM	30S ribosomal protein S13
NP_252932.1	4757095	4756979	-	PA4242	rpmJ	50S ribosomal protein L36
NP_252933.1	4758452	4757124	-	PA4243	secY	preprotein translocase SecY
NP_252934.1	4758887	4758453	-	PA4244	rplO	50S ribosomal protein L15
NP_252935.1	4759067	4758891	-	PA4245	rpmD	50S ribosomal protein L30
NP_252936.1	4759570	4759070	-	PA4246	rpsE	30S ribosomal protein S5
NP_252937.1	4759924	4759574	-	PA4247	rplR	50S ribosomal protein L18
NP_252938.1	4760468	4759935	-	PA4248	rplF	50S ribosomal protein L6
NP_252939.1	4760872	4760480	-	PA4249	rpsH	30S ribosomal protein S8
NP_252940.1	4761367	4761062	-	PA4250	rpsN	30S ribosomal protein S14
NP_252941.1	4761920	4761381	-	PA4251	rplE	50S ribosomal protein L5
NP_252942.1	4762254	4761940	-	PA4252	rplX	50S ribosomal protein L24
NP_252943.1	4762635	4762267	-	PA4253	rplN	50S ribosomal protein L14
NP_252944.1	4762925	4762659	-	PA4254	rpsQ	30S ribosomal protein S17
NP_252945.1	4763119	4762928	-	PA4255	rpmC	50S ribosomal protein L29
NP_252946.1	4763532	4763119	-	PA4256	rplP	50S ribosomal protein L16
NP_252947.1	4764230	4763544	-	PA4257	rpsC	30S ribosomal protein S3
NP_252948.1	4764575	4764243	-	PA4258	rplV	50S ribosomal protein L22
NP_252949.1	4764863	4764588	-	PA4259	rpsS	30S ribosomal protein S19
NP_252950.1	4765701	4764880	-	PA4260	rplB	50S ribosomal protein L2
NP_252951.1	4766012	4765713	-	PA4261	rplW	50S ribosomal protein L23
NP_252952.1	4766611	4766009	-	PA4262	rplD	50S ribosomal protein L4
NP_252953.1	4767260	4766625	-	PA4263	rplC	50S ribosomal protein L3
NP_252954.1	4767654	4767343	-	PA4264	rpsJ	30S ribosomal protein S10
NP_252955.1	4769004	4767811	-	PA4265	tufA	Translocation elongation factor Tu

NP_252956.1	4771155	4769035	-	PA4266	fusA1	Translocation elongation factor G
NP_252957.1	4771656	4771186	-	PA4267	rpsG	30S ribosomal protein S7
NP_252958.1	4772127	4771756	-	PA4268	rpsL	30S ribosomal protein S12
NP_252959.1	4776478	4772279	-	PA4269	rpoC	DNA-directed RNA polymerase, beta' subunit/160 kD subunit
NP_252960.1	4780617	4776544	-	PA4270	rpoB	DNA-directed RNA polymerase, beta subunit/140 kD subunit
NP_252961.1	4781207	4780839	-	PA4271	rplL	50S ribosomal protein L7/L12
NP_252962.1	4781786	4781286	-	PA4272	rplJ	50S ribosomal protein L10
NP_252963.1	4782680	4781985	-	PA4273	rplA	50S ribosomal protein L1
NP_252964.1	4783111	4782680	-	PA4274	rplK	50S ribosomal protein L11
NP_253357.1	5236774	5237391	+	PA4668	lolB	outer membrane lipoprotein LolB precursor
NP_253755.1	5706552	5706800	+	PA5068	tatA	twin arginine translocase protein A
NP_253756.1	5706814	5707239	+	PA5069	tatB	sec-independent translocase
NP_253757.1	5707236	5708039	+	PA5070	tatC	transport protein TatC

List of relevant genes with their start and stop positions, transcription strand, name and description are indicated.

Table 7.2: Operon prediction (Price, Huang, Alm, & Arkin, 2005).

SysName 1	SysName2	Name1	Name2	bOp	pOp	Sep
PA1346	PA1347		ldcC	FALSE	0.121	84
PA3699	PA3700		lysS	FALSE	0.065	212
PA3700	PA3701	lysS	prfB	TRUE	0.773	181
PA4236	PA4237	katA	rplQ	FALSE	0.011	282
PA4237	PA4238	rplQ	rpoA	TRUE	0.92	44
PA4238	PA4239	rpoA	rpsD	TRUE	0.599	23
PA4239	PA4240	rpsD	rpsK	TRUE	0.977	17
PA4240	PA4241	rpsK	rpsM	TRUE	0.996	19
PA4241	PA4242	rpsM	rpmJ	TRUE	0.866	131
PA4242	PA4243	rpmJ	secY	TRUE	0.662	29
PA4243	PA4244	secY	rplO	TRUE	0.991	1
PA4244	PA4245	rplO	rpmD	TRUE	0.999	4
PA4245	PA4246	rpmD	rpsE	TRUE	0.999	3
PA4246	PA4247	rpsE	rplR	TRUE	0.999	4
PA4247	PA4248	rplR	rplF	TRUE	0.998	11
PA4248	PA4249	rplF	rpsH	TRUE	0.998	12
PA4249	PA4250	rpsH	rpsN	TRUE	0.871	190
PA4250	PA4251	rpsN	rplE	TRUE	0.995	14
PA4251	PA4252	rplE	rplX	TRUE	0.996	20
PA4252	PA4253	rplX	rplN	TRUE	0.997	13
PA4253	PA4254	rplN	rpsQ	TRUE	0.995	24
PA4254	PA4255	rpsQ	rpmC	TRUE	0.999	3
PA4255	PA4256	rpmC	rplP	TRUE	0.999	0
PA4256	PA4257	rplP	rpsC	TRUE	0.998	12
PA4257	PA4258	rpsC	rplV	TRUE	0.997	13
PA4258	PA4259	rplV	rpsS	TRUE	0.997	13

PA4259	PA4260	rpsS	rplB	TRUE	0.996	17
PA4260	PA4261	rplB	rplW	TRUE	0.998	12
PA4261	PA4262	rplW	rplD	TRUE	1	-3
PA4262	PA4263	rplD	rplC	TRUE	0.997	14
PA4263	PA4264	rplC	rpsJ	TRUE	0.976	83
PA4264	PA4265	rpsJ	tufA	TRUE	0.745	157
PA4265	PA4266	tufA	fusA1	TRUE	0.986	31
PA4266	PA4267	fusA1	rpsG	TRUE	0.946	31
PA4267	PA4268	rpsG	rpsL	TRUE	0.979	100
PA4268	PA4269	rpsL	rpoC	FALSE	0.331	152
PA4269	PA4270	rpoC	rpoB	TRUE	0.991	66
PA4270	PA4270.1	rpoB		FALSE	0.065	151
PA4667	PA4668		lolB	TRUE	0.95	5
PA4668	PA4669	lolB	ipk	TRUE	0.972	2
PA5065	PA5066	ubiB	hisI	FALSE	0.204	116
PA5066	PA5067	hisI	hisE	TRUE	0.999	-7
PA5067	PA5068	hisE	tatA	TRUE	0.638	26
PA5068	PA5069	tatA	tatB	TRUE	0.976	14
PA5069	PA5070	tatB	tatC	TRUE	0.997	-3
PA5070	PA5071	tatC		TRUE	0.949	-3

Name 1 and 2 indicate the first and second genes in the pair. bOp refers to the prediction whether the pair lies in the same operon. pOp is the probability of the pair lying in the same operon. Values near 1 or 0 are confident predictions of being in the same operon or not, while values near 0.5 are low-confidence predictions. Sep indicates the distance between the two genes in the pair.

Appendix 3: Expected sizes of PCR products

ldcC – 225 bases PCR product

5′CTTCAAGAATTCGGGTTACGCGCGTGCCGGCAAGTCTCTACCCTGAGCCGACCGGCGGGGTTC
GCCCGCCTGCTGCTCGGGATCAACAATGTATAAAGACCTCAAATTTCCCGTCCTCATCGTCCATC
GCGACATCAAGGCCGACACCGTTGCCGGCGAACGCGTGCGGGGCATCGCCCACGAACTGGAGCAG
GACGGCTTCAGCATTCT TGACTACGTAATCAC-3′

lpp – 161 bases PCR product

5′CTTCAAGAATTCGTATTGACCCCATAGACAGCTTCGTCGACGCCCGTCCCGGCCCCCTTGGGC
TTGCCGGACGGCTTATGTCATGATGGCGCCACCCTCGCAGGTTCAAGGCCGGCTTTCTTCCTCTA
TGAACAAATCCCTTGCGCTGACTACGTAATCAC-3′

lysS – 125bases PCR product

5′CTTCAAGAATTCGCTGTCTCTGGGAGCTACTCGGGACACGGTGTTTCCTCCGCTCGCGGACGA
TGGGGCATTGTTCGGACTTTTTCGCGTGGAATCAATCGGCCGCTATCTGACTACGTAATCAC-3′

tat – 206 bases PCR product

5′CTTCAAGAATTCGGGGTATTCCTGATCCTGCGCCGCTAGCGCCGCGCACGGCCACTAGGCCCG
CGCCGATAGCCAGTCGCGCTCCCGGCTGGCACACTACTCCCATTTCCGCCGGAAACGCGCGCAAC
GTACCGGCAACGAACGTGGAAAGACCATGAAAGACTGGCTGGATGAGATTCACTGGAACGCCGTG
ACCTACGTATGCAC-3′

spc – 134 bases PCR product

5′CTTCAAGAATTCGTAACGCGCGGAACTTCCATCACGTTCGCCAGCTGAAGCTCTTCTTTCAGC
TTGGGCGCGATTTCCTTCCGATAAATTTCTTTCAATCGTGCCATGGTGATCTACCTTGACGTCGA
CATCAG-3′

Appendix 4: Optimisation of PCR conditions and restriction of DNA

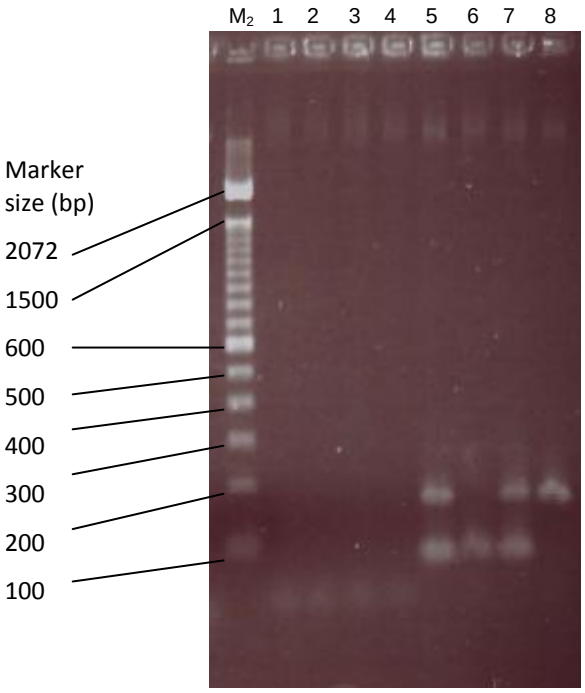

Figure 7.1: Agarose gel electrophoretic analysis of PCR reactions for P_{tat}. The reactions were performed at various magnesium chloride concentrations of 0.25mM (lanes 1-4) and 0.75mM (lanes 5-8). The reactions were also performed at various annealing temperatures of 53^{o}C (lane 1, 5), 56^{o}C (lane 2, 6), 58^{o}C (lane 3, 7) and 60^{o}C (lane 4, 8).

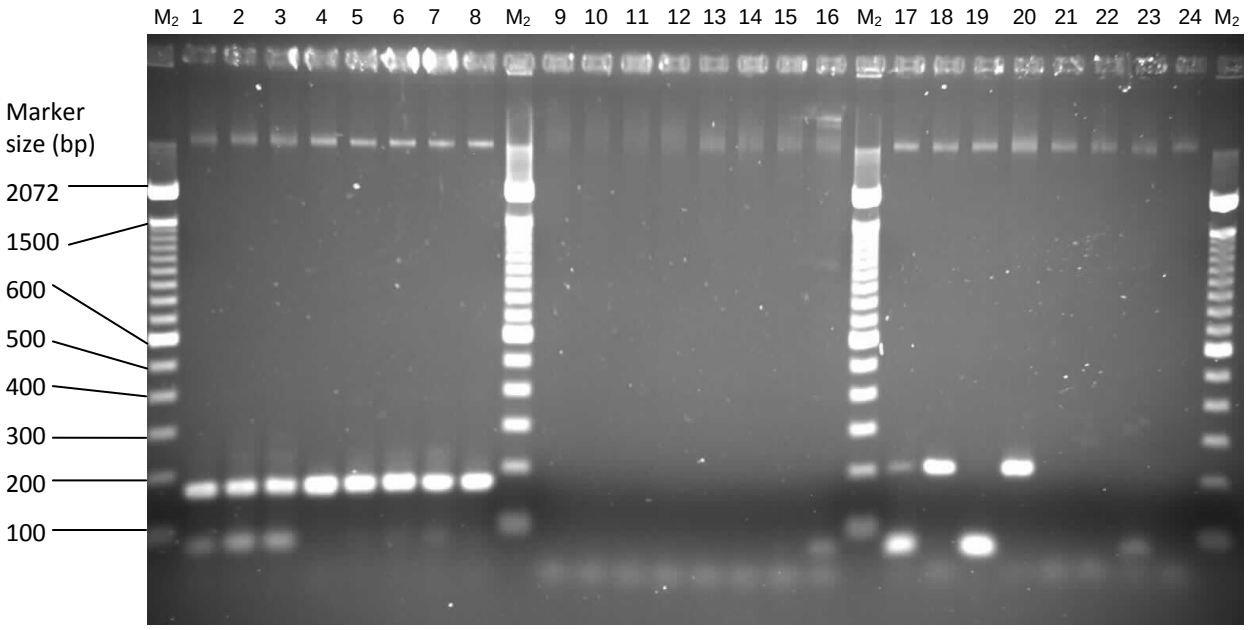

Figure 7.2: Agarose gel electrophoretic analysis of PCR reactions for P_{tat} and P_{lpp}. PCR reactions for P_{tat} and P_{lpp} are represented in lanes 1-8 and 9-24 respectively. The reactions were performed at various magnesium chloride concentrations of 0.25mM (lanes 9-12), 0.75mM (lanes 13-16), 1mM (lanes 1-4, 17-20) and 1.5mM (lanes 5-8, 21-24). The reactions were also performed at various annealing temperatures of 53°C (lane 1, 5), 56°C (lane 2, 6), 58°C (lane 3, 7) and 60°C (lane 4, 8) for P_{tat}, and 48°C (lane 9, 13, 17, 21), 50°C (lane 10, 14, 18, 22), 52°C (lane 11, 15, 19, 23) and 54°C (lane 12, 16, 20, 24) respectively for P_{lpp}.

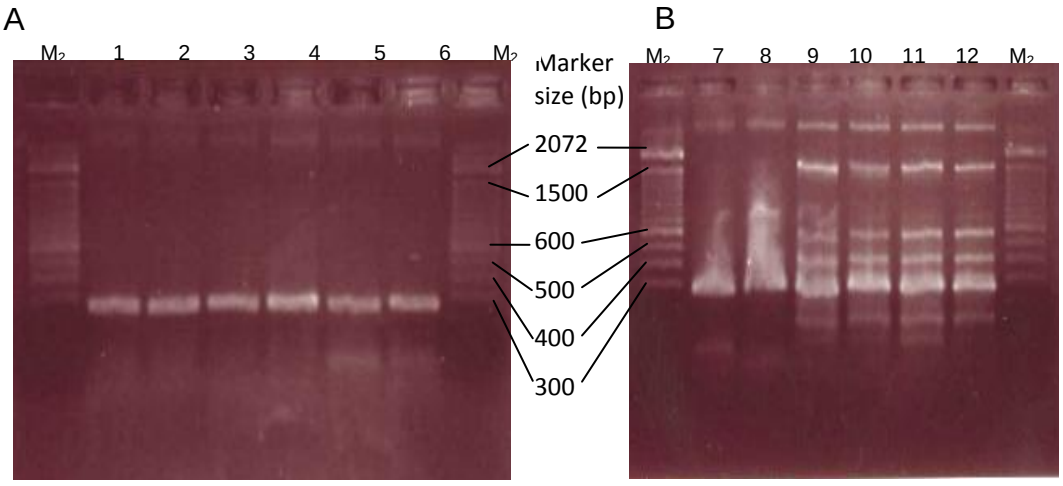

Figure 7.3: Agarose gel electrophoretic analysis of PCR reactions for P_{ldcC}. The reactions were performed at various magnesium chloride concentrations of 0.75mM (lanes 1-4), 1mM (lanes 5-8) and 1.5mM (lanes 9-12). The reactions were also performed at various annealing temperatures of 50°C (lane 1, 5, 9, 13), 52°C (lane 2, 6, 10, 14), 54°C (lane 3, 7, 11, 15) and 56°C (lane 4, 8, 12, 16).

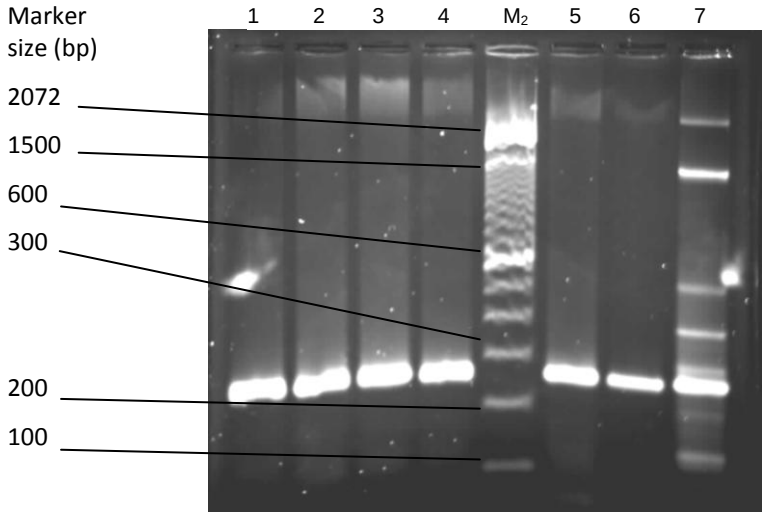

Figure 7.4: Agarose gel electrophoretic analysis of PCR reactions for P_{ldcC}. The reactions were performed at magnesium chloride concentrations of 0.75mM (lanes 1-3), 1mM (lanes 4-6) and 1.5mM (lanes 7);and annealing temperatures of 50^{o}C (lane 1, 4, 7), 52^{o}C (lane 2, 5) and 54^{o}C (lane 3, 6).

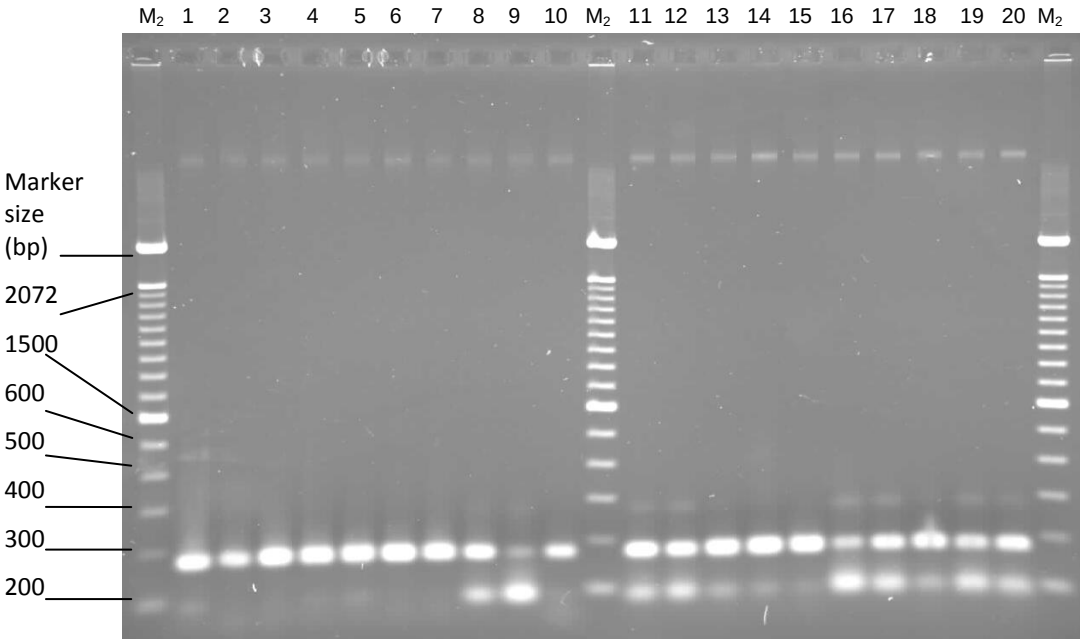

Figure 7.5: Agarose gel electrophoretic analysis of PCR reactions for P_{tat}. The reactions were performed at various magnesium chloride concentrations of 1mM (lanes 1- 10) and 1.5mM (lanes 11- 20). The reactions were also performed at various annealing temperatures of 53^{o}C (lane 1, 11), 54^{o}C (lane 2, 12), 56^{o}C (lane 3, 13), 58^{o}C (lane 4, 14), 60^{o}C (lane 5, 15), 62^{o}C (lane 6, 16), 64^{o}C (lane 7, 17), 66^{o}C (lane 8, 18), 68^{o}C (lane 9, 19) and 70^{o}C (lane 10, 20).

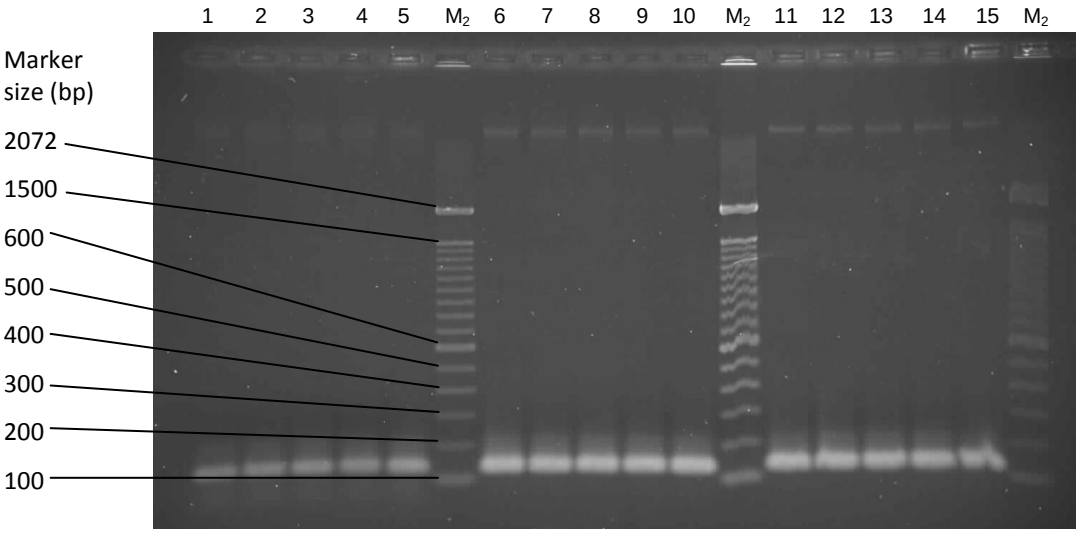

Figure 7.6: Agarose gel electrophoretic analysis of PCR reactions for P_{spc}. The reactions were performed at various magnesium chloride concentrations of 0.75mM (lanes 1-5), 1mM (lanes 6-10) and 1.5mM (lanes 11-15). The reactions were also performed at various annealing temperatures of 50^{o}C (lane 1, 6, 11), 52^{o}C (lane 2, 7, 12), 54^{o}C (lane 3, 8, 13), 56^{o}C (lane 4, 9, 14) and 58^{o}C (lane 5, 10, 15).

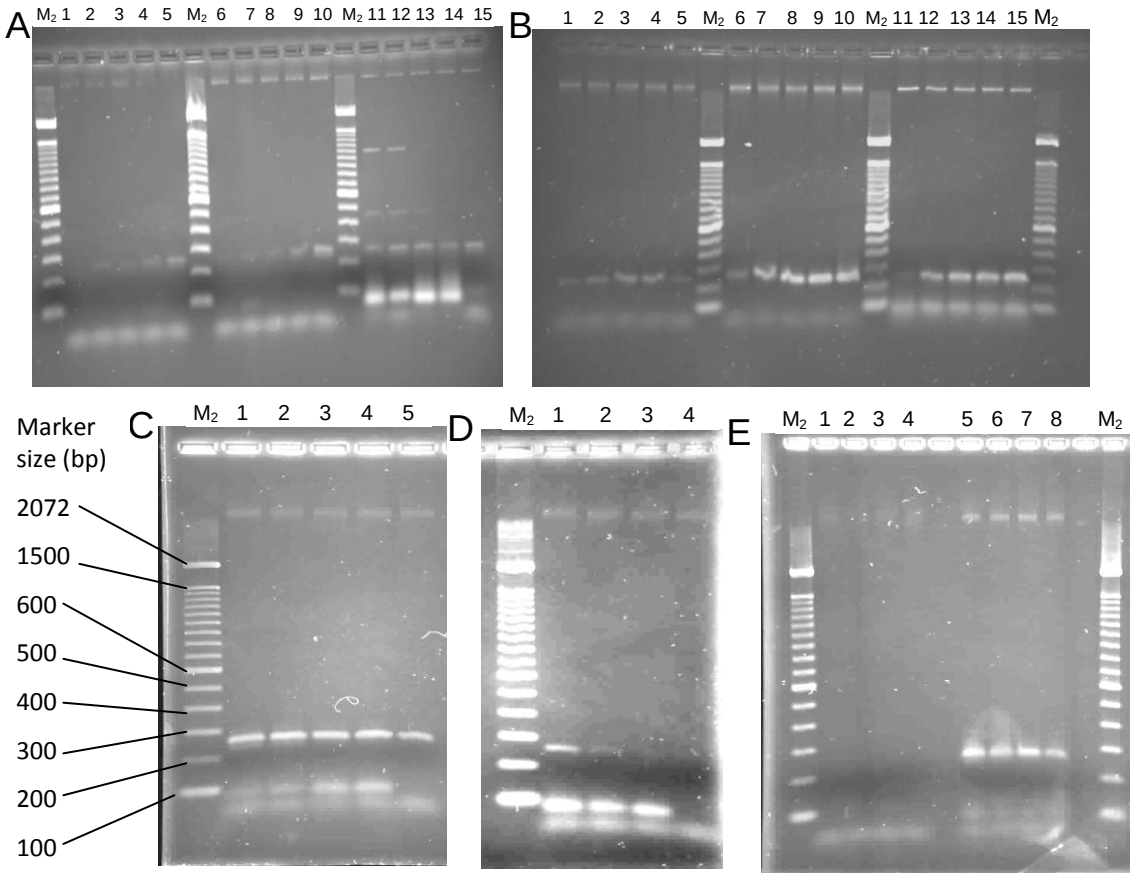

Figure 7.7: Agarose gel electrophoretic analysis of PCR reactions for P_{lysS}. The reactions were performed at various magnesium chloride concentrations and annealing temperatures. Gel A represents reactions performed using magnesium chloride concentrations of 0.75mM (lanes 1-5), 1mM (lanes 6-10) and 1.5mM (lanes 11-15), and annealing temperatures of 50^{o}C (lane 1, 6, 11), 52^{o}C (lane 2, 7, 12), 54^{o}C (lane 3, 8, 13), 56^{o}C (lane 4, 9, 14) and 58^{o}C (lane 5, 10, 15). Gel B represents reactions performed using magnesium chloride concentrations of 0.75mM (lanes 1-5), 1mM (lanes 6-10) and 1.5mM (lanes 11-15), and annealing temperatures of 56^{o}C (lane 1, 6, 11), 58^{o}C (lane 2, 7, 12), 60^{o}C (lane 3, 8, 13), 62^{o}C (lane 4, 9, 14) and 64^{o}C (lane 5, 10, 15). Gel C represents reactions performed using magnesium chloride concentrations of 1mM (lanes 1-5) at various annealing temperatures of 62^{o}C (lane 1), 64^{o}C (lane 2), 66^{o}C (lane 3), 68^{o}C (lane 4) and 70^{o}C (lane 5). Gel D represents reactions performed using magnesium chloride concentrations of 1mM (lanes 1-5) at various annealing temperatures of 68^{o}C (lane 1), 70^{o}C (lane 2), 72^{o}C (lane 3) and 74^{o}C (lane 4). Gel E represents reactions performed using magnesium chloride concentrations of 0.75mM (lanes 1-4) and 1.5mM (lanes 5-8), and annealing temperatures of 66^{o}C (lane 1, 5), 68^{o}C (lane 2, 6), 70^{o}C (lane 3, 7) and 72^{o}C (lane 4, 8).

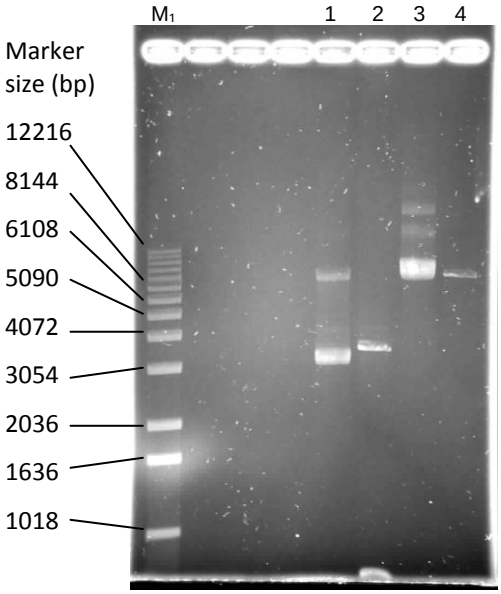

Figure 7.8: Agarose gel electrophoretic analysis for restriction of plasmids pBR322 and pSB417. Undigested and digested plasmid pBR322 are represented in lanes 1 and 2 respectively. Undigested and digested plasmid pSB417 are represented in lanes 3 and 4 respectively.

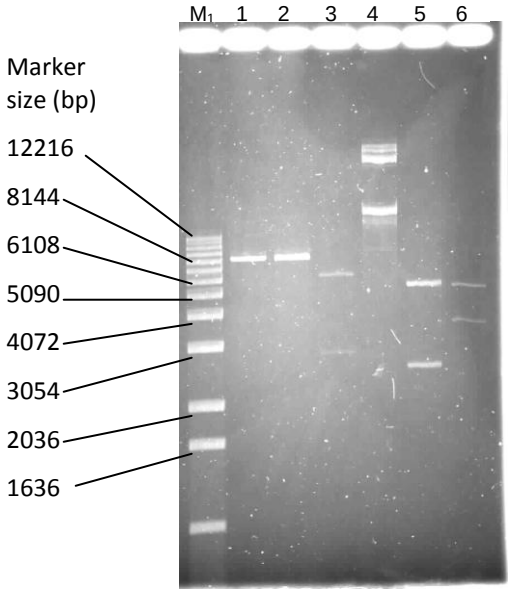

Figure 7.9: Agarose gel electrophoretic analysis for restriction of plasmids pBR322, pSB417 and pBR322-*lux*. Plasmid pSB417 digested with SalI and PstI in two separate reactions are represented in lane 1 and 2 respectively. Double digestion of plasmid pBR322-*lux* with SalI and PstI is represented in lanes 3. Undigested plasmid pBR322-*lux* is represented in lane 4. Plasmid pSB417 digested with EcoRI and BamHI in a double digest is represented in lane 5 while plasmid pBR322 digested in the same manner is represented in lane 6. Restriction digests were performed for 30 minutes.

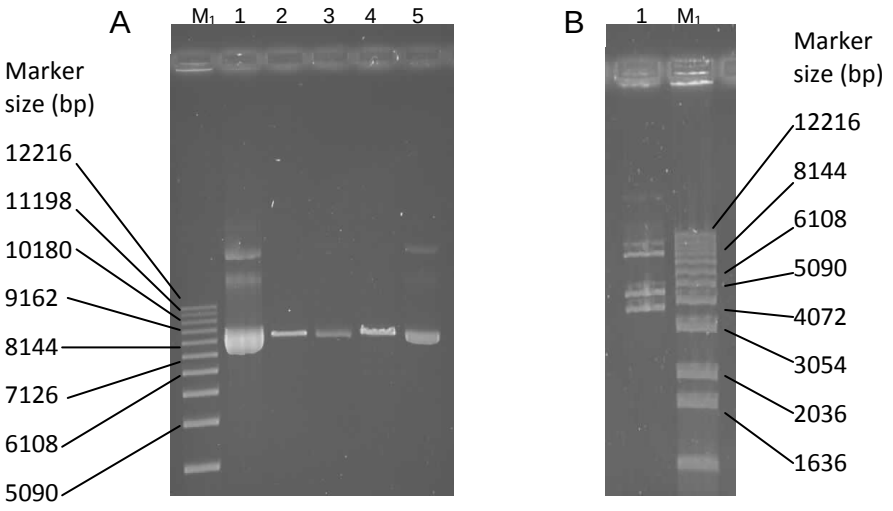

Figure 7.10: Agarose gel electrophoretic analysis for restriction of plasmid pSB417. Figure A: Undigested plasmid pSB417 is represented in lane 2 and 5. Plasmid pSB417 digested with SalI is represented in lane 2 and subsequently digested with PstI is represented in lane 3. Plasmid pSB417 digested with PstI is represented in lane 4. Figure B: A double digest of plasmid pSB417 with SalI and XbaI is represented in lane 1.

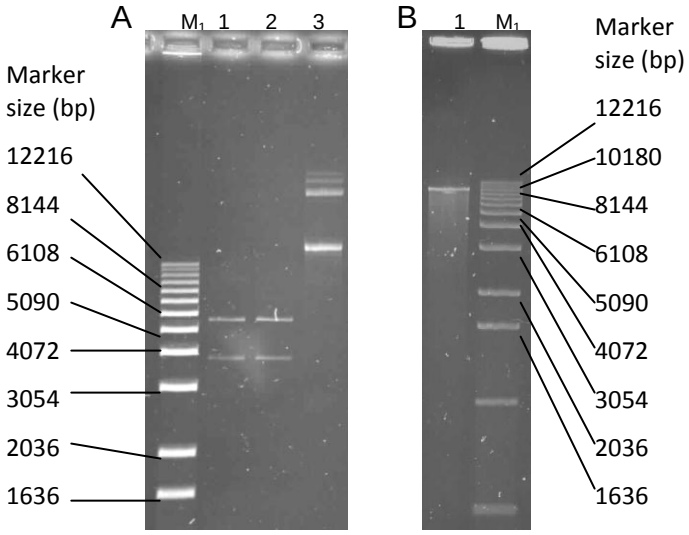

Figure 7.11: Agarose gel electrophoretic analysis for restriction of the promoterless plasmid pBR322-*lux*. Double digest of the plasmid with EcoRI and BamHI is represented in lanes 1 and 2. Undigested plasmid is represented in lane 3. Plasmid digested with SnaBI is represented in lane 1 of figure B.

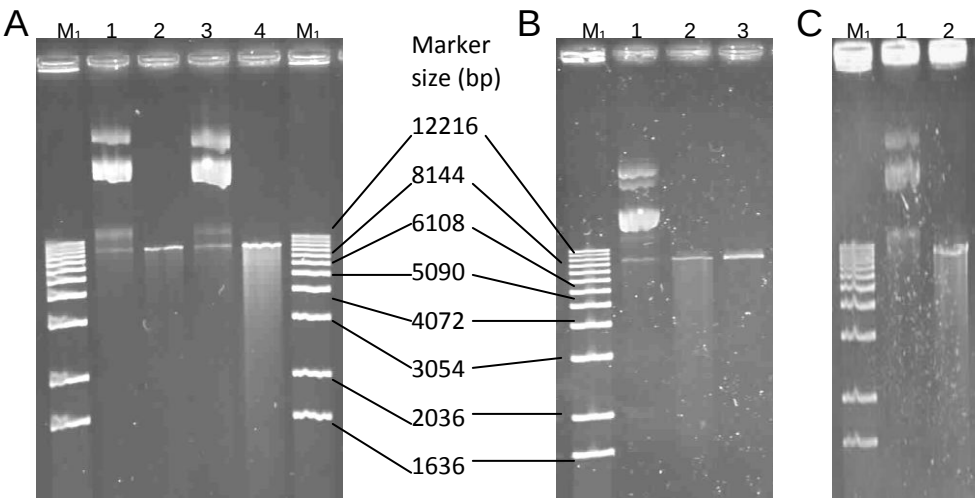

Figure 7.12: Confirmative agarose gel electrophoretic analysis for restriction of the promoterless plasmid pBR322-*lux*. A: Undigested plasmid is represented in lane 1 and 3. Plasmid digested with SnaBI and EcoRI is represented in lane 2 and 4 respectively. B: Undigested plasmid represented in lane 1. Successive digestions of plasmid with SnaBI first and then EcoRI is represented in lanes 2 and 3 respectively. C: Undigested plasmid is represented in lane 1. Double digest of the plasmid with EcoRI and SnaBI is represented in lane 2.

Appendix 5: Expected DNA sequence of promoterless pME-*lux*

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taacatcgttgctgctccataacatcaaacatcgacccacggcgtaacgcgcttgctgcttggatgcccgaggcatagactgtacaaaaaa
acagtcataacaagccatgaaaaccgccactgcgccgttaccaccgctgcgttcggtcaaggttctggaccagttgcgtgagcgcatacg
ctacttgcattacagtttacgaaccgaacaggcttatgtcaattcg

Replication protein

LacZ α gene RBS for LacZ gene

Multiple cloning site (pUCP24): EcoRI-SmaI-BamHI-SalI-PstI-HindIII

rrnB terminator

Gentamicin resistance gene RBS for gentamicin resistance gene

Upper case: *Lux* cassette
Lower case: pME4510 plasmid

*Lux*E *Lux*B *Lux*A *Lux*D *Lux*C

Appendix 6: DNA sequencing of constructs in *E. coli.*

Promoterless pME-*lux* sequence (EcoRI and SnaBI junction)

AnGaaTtGAatgTTatGCaCCGTAaTTCGTTATTTCCATTACAATCAATAATGTTTTTTACATGAgannccnnnnnann
ngGCAGGTAAACACTATTATCACCAAAATTAATGGATTGCACTAAATCATCACCTTCGGGAAAGATTTCAACC
TGGCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTATCCTCCAAGCCTGAATTCCTGGCAGTTTATGG
CGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGT
CCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTC
GTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCG
GTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGT
GAGCAAAAGGCCAGCAAAAGG

Promoterless pME-*lux* sequence (BamHI junction)

tncgGaAntTGTCCTACTCaggCTTGGGCTGCAgGTCGAcgGATCcgTCGACCTGCAgGcCccnnnnnannnaaACG
CtTCGGTTAAGCtTAAAGCACACcCTTTCTGcGTCCTcgTATTGACGCGACGTAAAATTTCAACGAGCACGCC
GGGATACTTACCATATTCTCTGCTAATTATCCCGACATCATCGGTAACAATAAATGCTGGATAACTGGTTGC
TGACGCATCCATATAACTCATCAACCCCGGCGTTCCATCAGGTACAGGTTTCAACGTTTCAGGATCAAGCG
CTCGCGCATATACCCACGGCGGAACATGTTTACGCTGCATTTCATCCTCAAAGAAACAAGTGTTGAGTTCA
ACTTGATTAAATATATCTCGGATCTGACTAATATCACTGAGATTGAAAGTATCAAATAAAAGATGATTGAAAT
CATCACGTTTCAGAGATTCTTTTTCGTAACTTTTCCAGCCGCCTCCGGTTATGATATAAAGGCTTTTATCTCC
AGAAAATGAGATTTTTTTATCTTTCATATAATGGCAGAGTAAATAAATAAAGTATGGCGAACCAATAAGACAA
AGATCTTTCCCTTGATTTTTTATTCGTTCAAGACTATTCAATgTTTTTAanaaAATCTATTcggttcTTCTgntacgggt
AAATGTCGnaaggATATAA

Spc3-pME-*lux* sequence (EcoRI and SnaBI junction)

GanTtGaATGTTatgCaCCGTAaTTCGTTATTTCcaTTACAATCAATAATGTTTTTTACAtgnanncCCnnnnnnaannn
ggCAGGTAAACACTATTATCACCAAAATTAATggaTTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCT
GGCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGGTAGATCACCATGGCACGATTGAAAGAA
ATTTATCGGAAGGAAATCGCGCCCAAGCTGAAAGAAGAGCTTCAGCTGGCGAACGTGATGGAAGTTCCGC
GCGTTACGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAAC
GTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAA
AGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGC
GCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
AGGGGATAACGCAGG

Spc5-pME-*lux* sequence (EcoRI and SnaBI junction)

TgaatgTTnngcaCCGTAaTTCGTTATTtCCATTACAATCAATAATGTTTTTTACATGAGngccnnnnnnaaannngCAG
GTAAACACTATTATCACCAAAATTAATggaTTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTGGCCG
TTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGGTAGATCACCATGGCACGATTGAAAGAAATTTAT
CGGAAGGAAATCGCGCCCAAGCTGAAAGAAGAGCTTCAGCTGGCGAACGTGATGGAAGTTCCGCGCGTT
ACGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAA
ATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCC
CAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCG
GTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGG
GATAACGCaggAAAGA

Spc9-pME-*lux* sequence (EcoRI and SnaBI junction)

CnngtTaTGCaCcgTAaTTCGTTATTtCCATTACAATCAATAATGTTTTTTACATGagagCCCnnnnnnaaannngGCAG
GTAAACACTATTATCACCAAAATTAATggaTTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTGGCCG
TTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAAGGTAGATCACCATGGCACGATTGAAAGAAATTTA

TCCGAAGGAAATCGCGCCCAAGCTGAAAGAAGAGCTTCAGCTGGCGAACGTGATGGAAGTTCCGCGCGTT
ACGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAA
ATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCC
CAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCG
GTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATAnggTTATCCACAGAATCAGGGG
GATAACGcagGAAAG

LdcC8-pME-*lux* sequence (EcoRI and SnaBI junction)

GaatGaCatgTTatGCaCCGTAaTTCGTTATTTCCATTACAATCAATAATGTTTTTTACATGagancCCnnnnnnnnnn
ngCAGGTAAACACtatTATCACCAAAATTAATggaTTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTG
GCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAAGAGAATGCTGAAGCCGTCCTGCTCCAGCT
CGTGGGCGATGCCCCGCACGCGTTCGCCGGCAACGGTGTCGGCCTTGATGTCGCGATGGACGATGAGGA
CGGGAAATTTGAGGTCTTTATACATTGTTGATCCCGAGCAGCAGGCGGGCGAACCCCGCCGGTGGGCTCA
GGGTAGAGAGTTGCCGGCACGCGCGTAACCCGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCA
CCCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTC
ACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGG
GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGG
CGGTAATA

LdcC4b-pME-*lux* sequence (EcoRI and SnaBI junction)

GanTtGAatgntTGCaCCGTAaTTCGTTATTTCcaTTACAATCAATAATGTTTTTTACATGAgannccnnnnnnannnGG
CAGGTAAACACTAtTATCACCAAAATTAATGGATTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTGG
CCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAAGAGAATGCTGAAGCCGTCCTGCTCCAGCTC
GTGGGCGATGCCCCGCACGCGTTCGCCGGCAACGGTGTCGGCCTTGATGTCGCGATGGACGATGAGGAC
GGGAAATTTGAGGTCTTTATACATTGTTGATCCCGAGCAGCAGGCGGGCGAACCCCGCCGGTGGGCTCAG
GGTAGAGAGTTGCCGGCACGCGCGTAACCCGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCAC
CCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCA
CCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGG
CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGC
GGTAATA

Lys2-pME-*lux* sequence (EcoRI and SnaBI junction)

TtGCaccgTatTCGnttTTCATTacaTCAATAATGTTTTttnATGAgannccnnnnnannngGCAGGTAAACACTATTATCA
CCAAAATTAATgGATTGCACTAAATCATCACCTTCGGGAAAGATTtCAACCTGGCCGTTAATAATGAATGAAA
TTTTTTTAGTCATACGTAGTCAGATAGCGGCCGATTGATTCCACGCGAAAAAGCCCGAACAATGCCCCATC
GTCCGCGAGCGGAGGAAACACCGTGCCCCGAGTAGCTCCCAGAGACAGCGAATTCCTGGCAGTTTATGG
CGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGT
CCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTC
GTTTTATTTGATGCCTGGGGCtTCCTCGCTCaCTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCG
GtATCAGCTCACTcnaAGGCGGTa

Lys3-pME-*lux* sequence (EcoRI and SnaBI junction)

ATtnnCaTGTtaTggCACCGTAaTTCGTTATTTCcATTACAATCAATAATGTTTTTTACATGagnncncnnnnnnaannt
gGCAGGTAAACACTAtTATCACCAAAATTAATggaTTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTG
GCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGATAGCGGCCGATTGATTCCACGCGAAAAA
GCCCGAACAATGCCCCATCGTCCGCGAGCGGAGGAAACACCGTGCCCCGAGTAGCTCCCAGAGACAGCG
AATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAATC
CGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAG
TCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATA
ACGCAGGAAAGAA

Lys6-pME-*lux* sequence (EcoRI and SnaBI junction)

GgaatGAaTgnttGCaCCGTAaTTCGTTATTTCCATTACAATCAATAATGTTTTTTACATGAGagCCCnnnnnnaann
gGCAGGTAAACACTATTATCACCAAAATTAATGGATTGCACTAAATCATCACCTTCGGGAAAGATTTCAACC
TGGCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGATAGCGGCCGATTGATTCCACGCGAAA
AAGCCCGAACAATGCCCCATCGTCCGCGAGCGGAGGAAACACCGTGCCCCGAGTAGCTCCCAGAGACAG
CGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAA
TCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCC
AGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGG
TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGA

Lpp4-pME-*lux* sequence (EcoRI and SnaBI junction)

GgnaTtGAaTGtttGCaCCGTaATTCGTTATTtCCATTACAATCAATAATGTTTTTTACATGAGagcccnnnnnnaannn
GCAGGTAAACACTATTATCACCAAAATTAATGGATTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCT
GGCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGCGCAAGGGATTTGTTCATAGAGGAAGAA
AGCCGGCCTTGAACCTGCGAGGGTGGCGTCATCATGACATAAGCCGTCCGGCAAGCCCAAGGGGGCCGG
GACGGGGCGTCGACGAAGCTGTCTATGGGGTCAATACGAATTCCTGGCAGTTTATGGCGGGCGTCCTGC
CCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGA
GCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATG
CCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATA

Lpp8-pME-*lux* sequence (EcoRI and SnaBI junction)

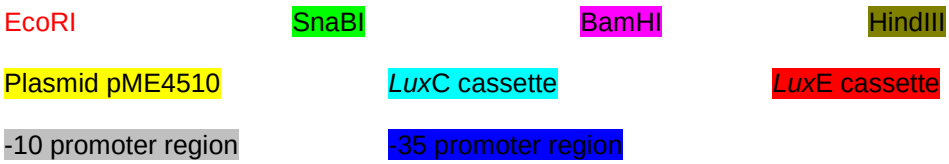
GGnaTtGaCaTGtTatGCaCCGTAaTTCGTTATTTCcaTTACAATCAATAATGTTTTTTACATGagcnnncnnnnnaann
gGCAGGtAAACACTAttaTCACCAAAATTAATgGATTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTG
GCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGCGCAAGGGATTTGTTCATAGAGGAAGAAA
GCCGGCCTTGAACCTGCGAGGGTGGCGTCATCATGACATAAGCCGTCCGGCAAGCCCAAGGGGGCCGG
GACGGGGCGTCGACGAAGCTGTCTATGGGGTCAATACGAATTCCTGGCAGTTTATGGCGGGCGTCCTGC
CCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGA
GCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATG
CCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATAC

Tat9-pME-*lux* sequence (EcoRI and SnaBI junction)

AnGnaTtGaaTgttaTGCaCCGTaATTCGTTATTTCCATTACAATCAATAATGTTTTTTACATGanancnnnnnnnnnnn
nngCAGGTAAACACTATTAtCACCAAAATTAATggaTTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCT
GGCCGTTAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGGCGTTCCAGTGAATCTCATCCAGCCAGT
CTTTCATGGTCTTTCCACGTTCGTTGCCGGTGCGTTGCCCGCGTTTCCGGCGGAAATAGGGGTAGTGTGC
CAGCCGTGGACGCGACTGGCTATCGGCGCAGGATCAGGAATACCCCGAATTCCTGGCAGTTTATGGCGG
GCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTA
CTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTT
TATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTAT
CAGCTCACTCAAAG

Tat12-pME-*lux* sequence (EcoRI and SnaBI junction)

TnntTatGCaccggTAATTCGTTATTTCCaTTACAATCAATAAtggTTTTTTACATGAgngcCcnnnnnnaannnngCAGG
TAAACACTAtTATCACCAAAATTAATgncATTGCACTAAATCATCACCTTCGGGAAAGATTTCAACCTGGCCGT
TAATAATGAATGAAATTTTTTTAGTCATACGTAGTCAGGCGTTCCAGTGAATCTCATCCAGGTGCATACGTA
GTCAGGCGTTCCAGTGAATCTCATCCAGCCAGTCTTTCATGGTCTTTCCACGTTCGTTGCCGGTGCGTTGC
CCGCGTTTCCGGCGGAAATAGGGGTAGTGTGCCAGCCGTGGACGCGACTGGCTATCGGCGCAGGATCAG
GAATACCCCGAATTCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCCGTTGCTTCGCAA
CGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGA
AAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGGGCTTCCTCGCTCACTGACTCGCTG
CGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAT
CAGGGG


EcoRI
SnaBI
BamHI
HindIII
Plasmid pME4510
*Lux*C cassette
*Lux*E cassette
-10 promoter region
-35 promoter region

Appendix 7: Sequences of all plasmids in *P. aeruginosa*

pME-*lux* plasmid

CCTCTCAGGCGCCGCTGGTGCCGCTGGTTGGACGCCAAGGGTGAATCCGCCTCGATACCCTGATTACTCGCTTCCTGCGCCCTCTCAGGCGGCGATAGGGGACTGGTAAAACGGGGATTGCCCAGACGCCTCCCCCGCCCC
TTCAGGGGCACAAATGCGGCCCCAACGGGGCCACGTAGTGGTGCGTTTTTTGCGTTTCCACCCTTTTCTTCCTTTTCCCTTTTAAACCTTTTAGGACGTCTACAGGCCACGTAATCCGTGGCCTGTAGAGTTTAAAAAGGGAC
GGATTTGTTGCCATTAAGGGACGGATTTGTTGTTAAGAAGGGACGGATTTGTTGTTGTAAAGGGACGGATTTGTTGTATTGTGGGACGCAGATACAGTGTCCCCTTATACACAAGGAATGTCGAACGTGGCCTCACCCCCA
ATGGTTTACAAAAGCAATGCCCTGGTCGAGGCCGCGTATCGCCTCAGTGTTCAGGAACAGCGGATCGTTCTGGCCTGTATTAGCCAGGTGAAGAGGAGCGAGCCTGTCACCGATGAAGTGATGTATTCAGTGACGGCGGA
GGACATAGCGACGATGGCGGGTGTCCCTATCGAATCTTCCTACAACCAGCTCAAAGAAGCGGCCCTGCGCCTGAAACGGCGGGAAGTCCGGTTAACCCAAGAGCCCAATGGCAAGGGGAAAAGACCGAGTGTGATGATT
ACCGGCTGGGTGCAAACAATCATCTACCGGGAGGGTGAGGGCCGTGTAGAACTCAGGTTCACCAAAGACATGCTGCCGTACCTGACGGAACTCACCAAACAGTTCACCAAATACGCCTTGGCTGACGTGGCCAAGATGGA
CAGCACCCACGCGATCAGGCTTTACGAGCTGCTCATGCAATGGGACAGCATCGGCCAGCGCGAAATAGAAATTGACCAGCTGCGAAAGTGGTTTCAACTGGAAGGCCGGTATCCCTCGATCAAGGACTTCAAGTTGCGAG
TGCTTGATCCAGCCGTGACGCAGATCAACGAGCACAGCCCGCTACAGGTGGAGTGGGCGCAGCGAAAGACCGGGCGCAAGGTCACACATCTGTTGTTCAGTTTTGGACCGAAGAAGCCCGCCAAGGCGGTGGGTAAGG
CCCCAGCGAAGCGCAAGGCCGGGAAGATTTCAGATGCTGAGATCGCGAAACAGGCTCGCCCTGGTGAGACATGGGAAGCGGCCCGCGCTCGACTAACCCAGATGCCGCTGGATCTGGCCTAGAGGCCGTGGCCACCAC
GGCCCGGCCTGCCTTTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG
ACGTTGTAAAACGACGGCCAGTGAATTGTCCATTTTAGCCTCCTTAGCTCCTGAAAATCTCGTCGAAGCTCGGCGGATTTGTCCTACTCAAGCTTGGCTGCAGGTCGACGGATCCGTCGACCTGCAGGTCATCAACTATCAA
ACGCTTCGGTTAAGCTTAAAGCACACCCTTTCTGCGTCCTCGTATTGACGCGACGTAAAATTTCAACGAGCACGCCGGGATACTTACCATATTCTCTGCTAATTATCCCGACATCATCGGTAACAATAAATGCTGGATAACTG
GTTGCTGACGCATCCATATAACTCATCAACCCCGGCGTTCCATCAGGTACAGGTTTCAACGTTTCAGGATCAAGCGCTCGCGCATATACCCACGGCGGAACATGTTTACGCTGCATTTCATCCTCAAAGAAACAAGTGTTGA
GTTCAACTTGATTAAATATATCTCGGATCTGACTAATATCACTGAGATTGAAAGTATCAAATAAAAGATGATTGAAATCATCACGTTTCAGAGATTCTTTTTCGTAACTTTTCCAGCCGCCTCCGGTTATGATATAAAGGCTTT
TATCTCCAGAAAATGAGATTTTTTTATCTTTCATATAATGGCAGAGTAAATAAATAAAGTATGGCGAACCAATAAGACAAAGATCTTTCCCTTGATTTTTTATTCGTTCAAGACTATTCAATGTTTTAACAAAATCTATTCGTTC
TTCTGTTACGGTAAATGTCGTAGGATATAACAATTCCACCAAACTCATAACATATTTAAACCAAATATTATGAGCATTAAATCTATCTGGTCCCAAATTGACTAATTCTATTTGATGATCAAACCAACTACCAACATATTTCATG
CCATAACTCACAGAGCCTAAGAGTCTCTCAATACTTAATCTGTCACGCGCCACCTGACTTTTTAAACCATTCGTGCCGCTACTGGTAAACCAACTTTCAATCTCGTTTTCCTGAGAAGTTAATAAGCGAGTAAACTTAAAAACC
GATGTTGGGAATACAGGTATGTCATCAATTTCCGTAATATTGTCATCTACTTTGTGTGCCTGACAGTAGTGACGATATTCTCGACAATGTTTATAATGATTACGAAATGCATCAAGCACAAGTTTCTTTCTGATTTTTTCCTGC
TCGTCGTAAGACCACACTAATGGATCGCTCGAAAAAATCAAATCATCAATTTCTGAGCTTGCTGTAATTTCTTGTTTATCAACATATGAAGTCATACCTGTTTTACTCCTCAAGATAATATTAGAAAGTATGGCAGCACTGCTG
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GGTACTTCTTAATATTATCATCAACAATATTGATTACATTTTTTTGGCTCATCAAATCATTCATTGGTTCAAAGGACAGCAATACACTTTTCGCACCACACTTTTCAATTGCCAACTTAGCCGCAGTTATACACTCCGTATAATTT
CCGACAGCGTTTTCTGCAATTATTTCTTCAAGTTTATTTTCGAAATTTTCATTAGGGTGCATTTCAAGAACATAATCACTAATAAATGCACGCGTCTCTTGTTTAGCTTTATTACTATCTTCGTTATAGTTAACTAATATCATTAA
CTGATGGTCTATCTCTGATAGGTCAACGTCATATTTATCCGCAACGGCTTTATATCTTTCAGCATATTCATATCTAACATCATTAGAATCATCCCACTTAAAGATGAGAGGAATACCTTTTTTGGCCGCCCACTCAACAATATG
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AGAAATGGCATGACATCAGACTGGAAGAGCTTCATGGAAGCAATAATTTCGTCTACTGTTCCATTAGCTTCAAATCCACAACAAATATTTGATATTCCTGTAGCATCAATGTCTTTTTGAATTATGTCAATACATTCCTGCGGC
GTTCCCACGGGATTGATTTCGTAACTGTAATCAATACGGCGATTAGTATCTTTATGTCCTTTTAATACAAAGTCACGCCACTGCCCTTTATTGAAATCATAACCTCTTGTTTGGTCTGAATCATCAAAAATAGTCGTAGCATTCA
CATAAGAATCATACCAATGCCCCAGAAATTTCCGGAGGTCCGCCTGGCGTATAAGCATGGGGATTTACAGATATTTTAGGGAAGCTATAAAAATCGTTATCTGGATTACAATAGCCTGTTGTTAAAGCATCGTTAATGATTTCATAACACT
GCTCAAGTTGTGCTTTCTTTTCGTTAGTATTTATAATCCAACTTAATATCATCGGTAGGCCAAATTGAGCAGCCCACTCAGTCGTCGAAGCTGATTCAGCCACCACATAAACCGGTGCGCCACCTCTGCTATACGCCGCGGGG
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AGTAAATATGCAGCAGCGACATAAGGGTTACCAAGCAAACCAAACTCCGTGAAATGATGCTCCAGTAACCATACGGTATCAAAACCACACTCCTCAGAGATGCGACCTAATTTAACCAAACGTTTCATTACCTCTGTTTGAG
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GTCATGCGAACTTCCTAACAAAGAATATATCTTGCATCGATTACTACGAATATTTGATAACAATGTGATAACTTCATCTTGCTTGACCCAATTATCGTTATTTGCAGTAAAAGCAATAAACGGTATATCAAGATACATCATGTT
ATTAATTGTAGAAGCTAAATCTTCCCAACCAAAATCAAGACAATCTCTCGCAAAGACTTCAGCACCCAATTTATGGCCTTCAAAATCTAGATTATCCGGCAATTCATTAATGGGTAGACTGAGATAATCAAACCCTAAAGCTC
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CAATCAACCACTGCTAACAAGCTCTGCTTTCCTATAGACATTGTAAATTCATCAATTGTCCCTGAACTCAATCCAACGTGGTGAAGCGAATCATAGCGGATCACATGAAATCCATTCCGCGATAAATATTCCGCCAGACCAGC
AAAATGATCCATCCTGCGGGCAAAACCAGACGCAATAATAATGGCATTCTTTCTCTTTGGGCTGTTTTCTTCTGGCAGCGTTTCCCAAACATGAATTTTTTTATTTCCTTCAACACAAATAACGTGGTCGATGGTTTTATATTTT
GATTCATTTTCCATACTTTTACCTATTATGGGACAAATACAAGGAACTTATCTTCTTCCAGGAATCGAGTCTGTTCTATTTCAACCGCAACATCCTTAGCCGTATAGTTAGATGGCCTTTCATGAGAAATATATGTCACTAATCG
TTGCAACGGTCTCATTCCGTCATGAGATCCACCAACTCGAAATATGTTATTCATTCCTGCTTCTACAATCCTTTCCGCACCTTTTAATGCTAACGCATCTCGATATTTAAATGATGACTCCCAAGGAAAAATAGATATGGTTTGC
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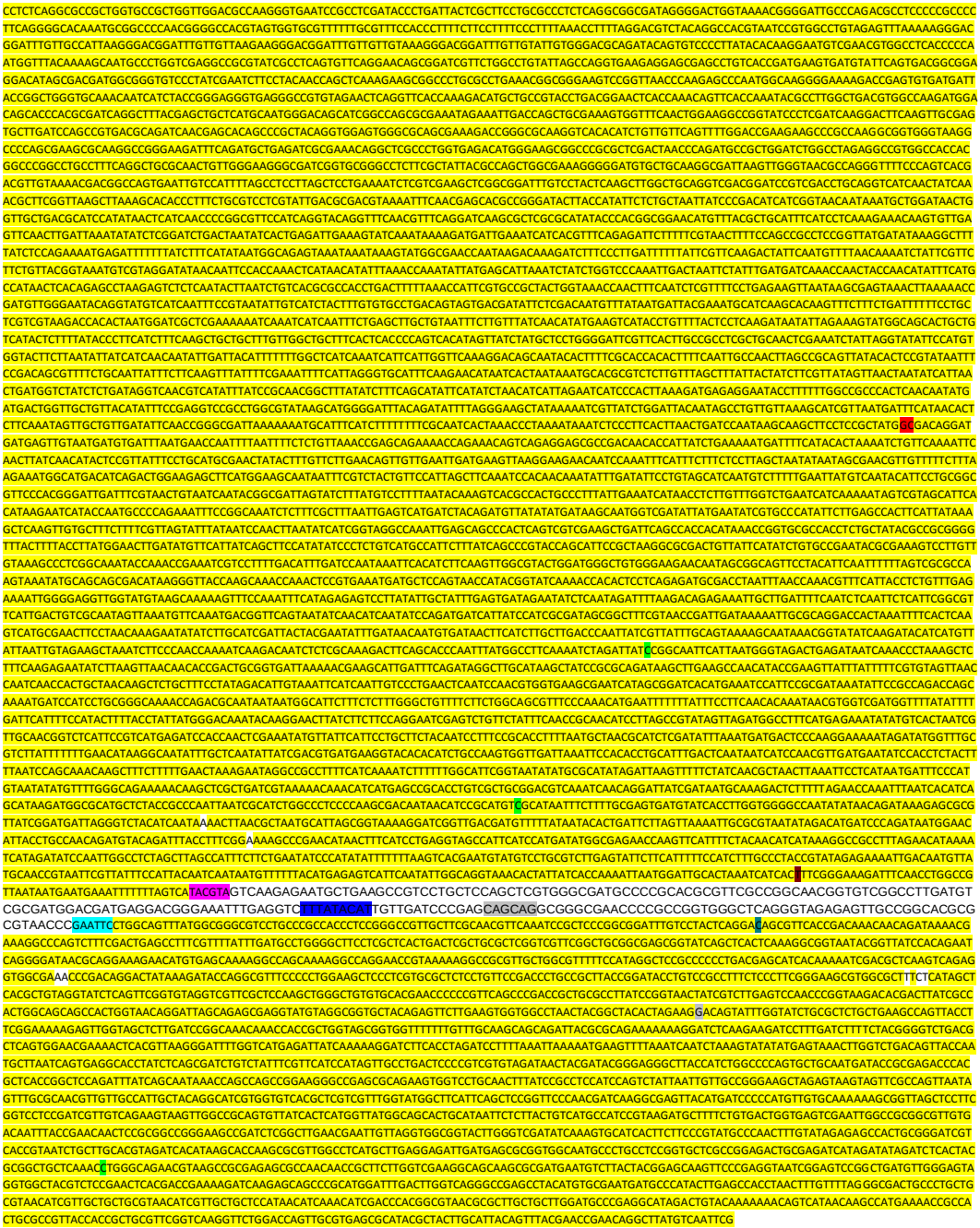

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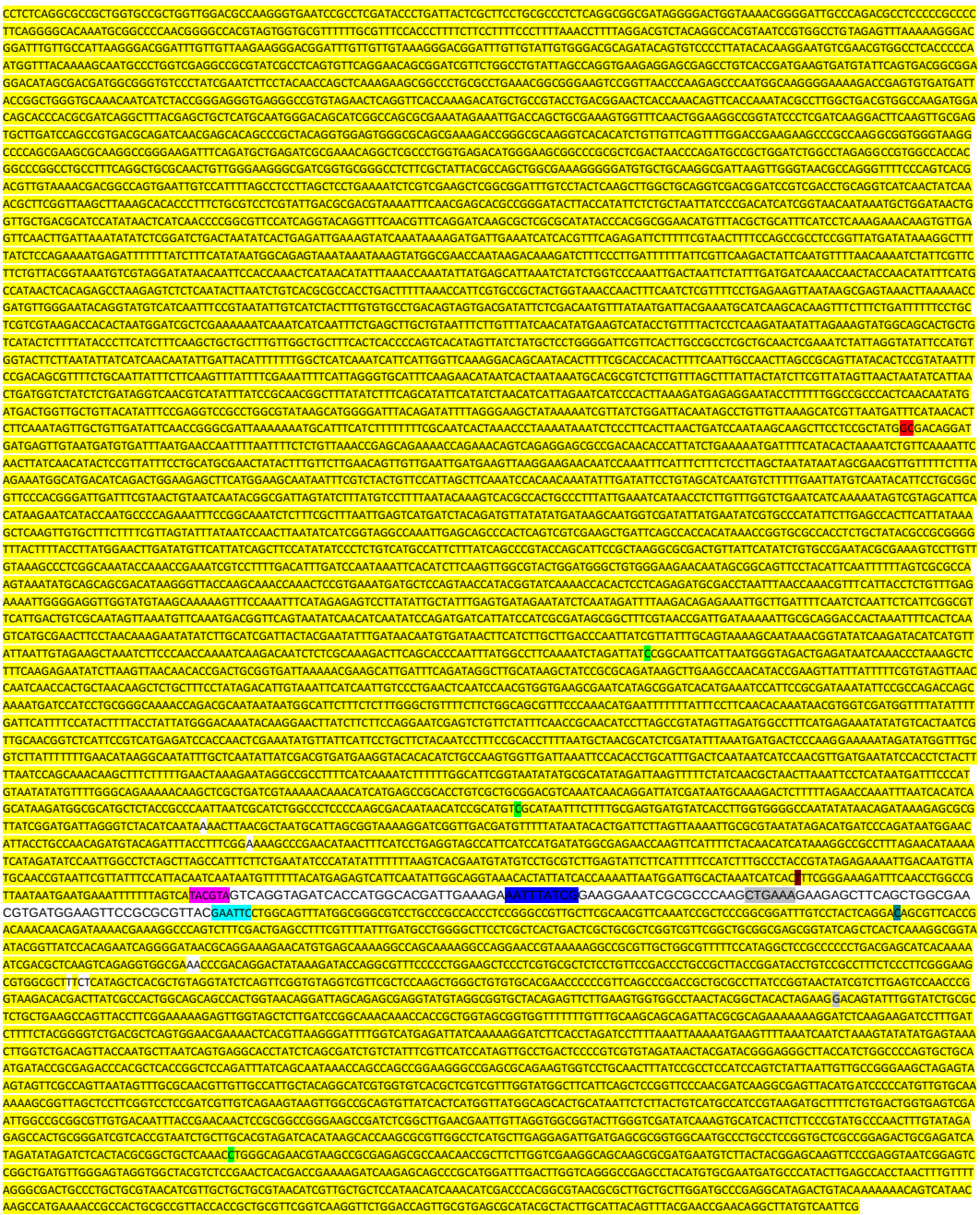

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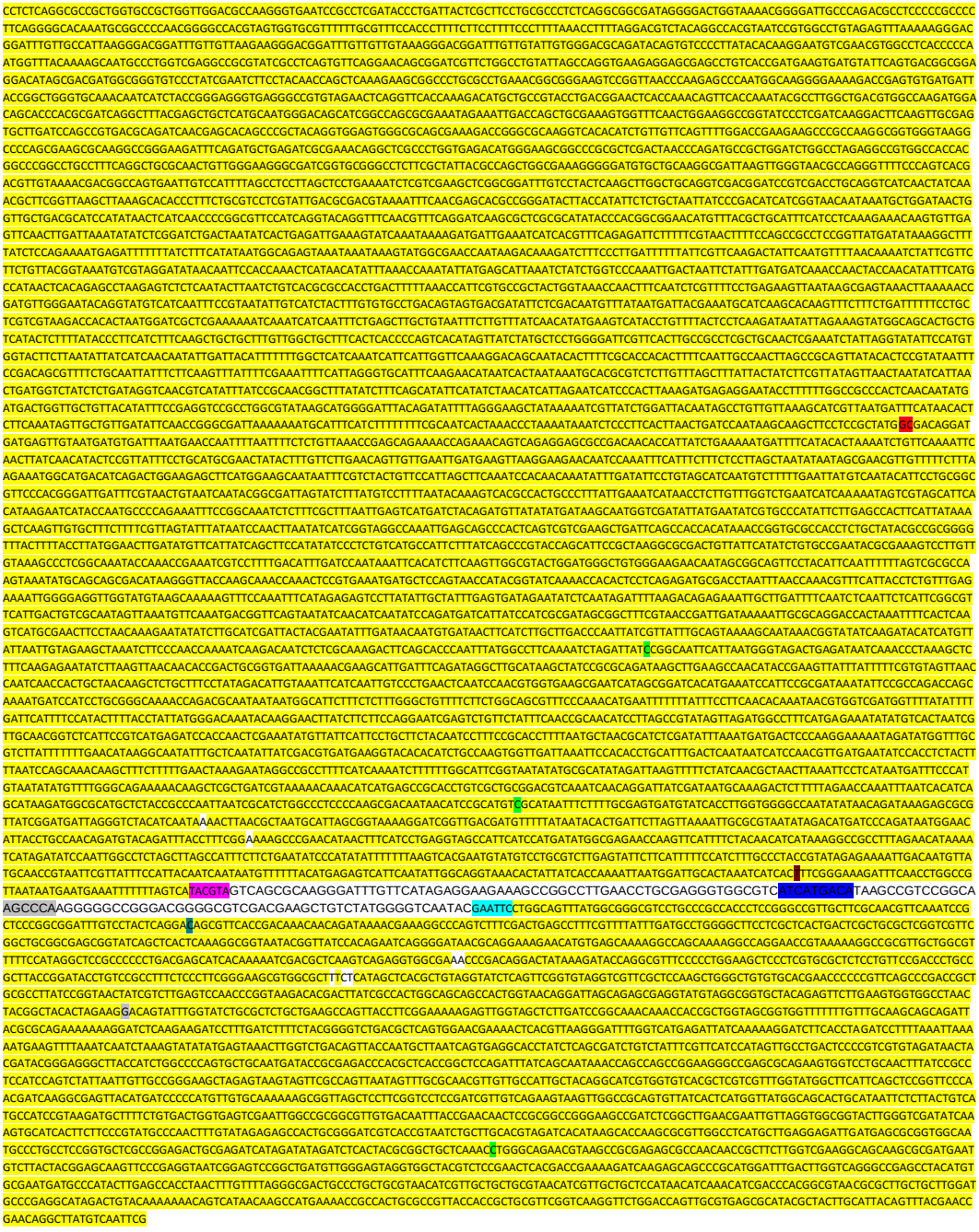

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GAACAGGCTTATGTCAATTCG

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CAGTTTACGAACCGAACAGGCTTATGTCAATTCG

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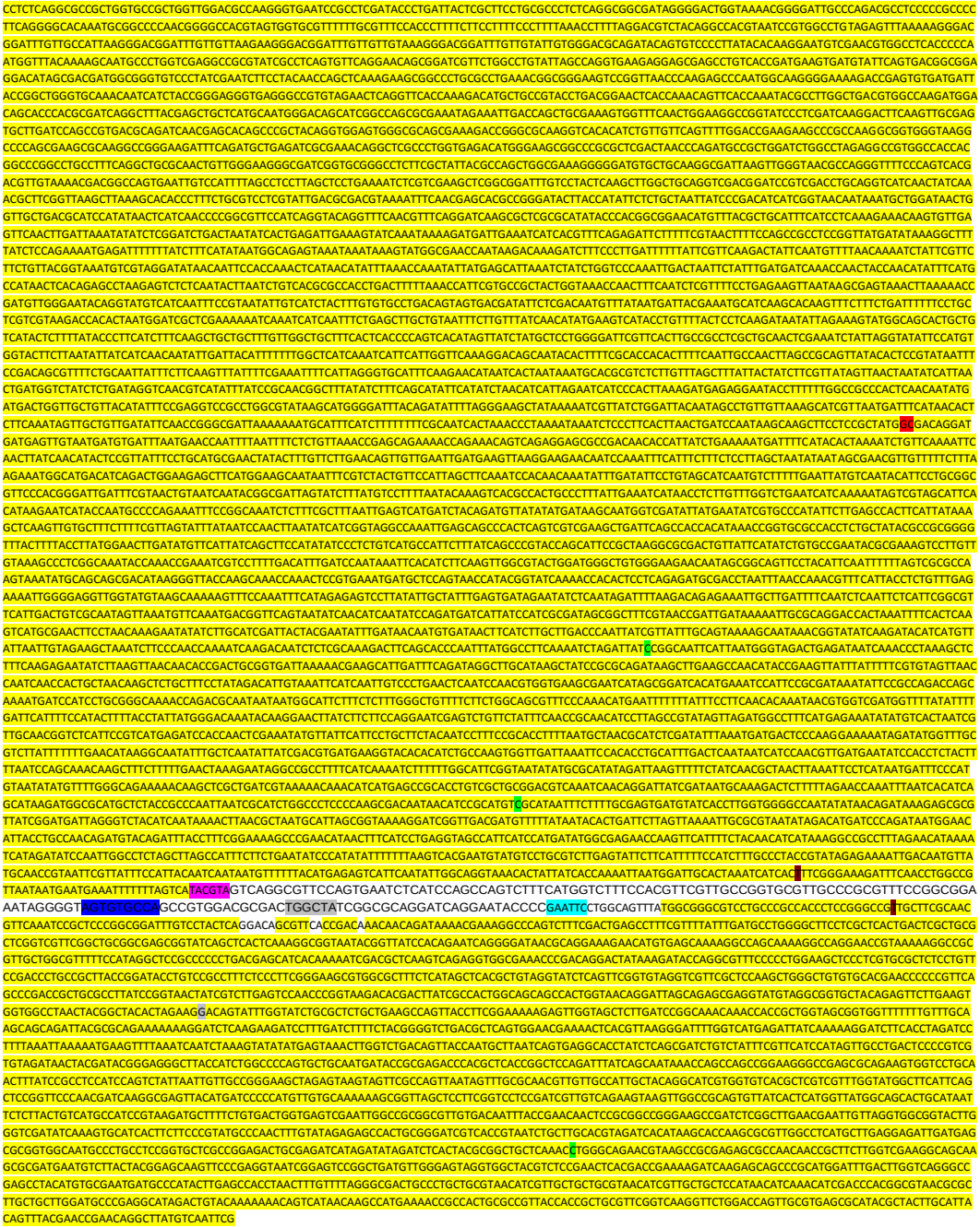
TatH14-pME-*lux*



TACGTA – SnaBI site GAATTC – EcoRI site Bases switched with each other

C converted to T C converted to G G converted to A T converted to C Addition of T

-35 Promoter sequence -10 Promoter sequence

Appendix 8: Validation

Normality and constant variance test

LysR25

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9943	0.9886	0.9828	0.2163

	Coefficient	Std. Error	t	P
a	9.2401	2.0090	4.5993	0.0100
b	0.3609	0.0752	4.8011	0.0086
x0	6.2140	1.3208	4.7047	0.0093

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.3696)
W Statistic= 0.9061 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.4383)

LysG25

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9877	0.9756	0.9634	0.2805

	Coefficient	Std. Error	t	P
a	7.4474	1.5832	4.7041	0.0093
b	0.3962	0.1110	3.5707	0.0234
x0	5.0918	1.2321	4.1327	0.0145

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.1281)
W Statistic= 0.8520 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.7810)

TatH5

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9930	0.9860	0.9790	0.2396

	Coefficient	Std. Error	t	P
a	8.8295	1.9052	4.6344	0.0098
b	0.3787	0.0842	4.4976	0.0108
x0	5.9787	1.2699	4.7082	0.0093

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.2942)
W Statistic= 0.8936 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.0956)

TatH9

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9881	0.9764	0.9646	0.2585

	Coefficient	Std. Error	t	P
a	7.9098	2.4671	3.2062	0.0327
b	0.3459	0.1063	3.2545	0.0312
x0	6.1210	1.9728	3.1027	0.0361

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.2802)
W Statistic= 0.8911 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.1815)

TatH14

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9927	0.9854	0.9781	0.2208

	Coefficient	Std. Error	t	P
a	7.0357	0.9006	7.8125	0.0014
b	0.4403	0.0881	4.9974	0.0075
x0	5.0301	0.6890	7.3004	0.0019

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.2213)
W Statistic= 0.8788 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.1815)

LppR3

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9865	0.9731	0.9597	0.2802

	Coefficient	Std. Error	t	P
a	16.8952	26.1381	0.6464	0.5532
b	0.2332	0.1091	2.1386	0.0992
x0	10.8524	11.1828	0.9705	0.3868

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.2759)
W Statistic= 0.8902 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.6019)

LppR4

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9914	0.9828	0.9742	0.2351

	Coefficient	Std. Error	t	P
a	7.0263	0.9868	7.1204	0.0021
b	0.4307	0.0948	4.5454	0.0105
x0	4.4543	0.7677	5.8021	0.0044

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.3443)
W Statistic= 0.9022 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.3884)

LdcC

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9835	0.9673	0.9510	0.2820

	Coefficient	Std. Error	t	P
a	15.6450	27.1065	0.5772	0.5948
b	0.2274	0.1198	1.8987	0.1304
x0	11.3584	12.8547	0.8836	0.4268

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.3543)
W Statistic= 0.9038 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.8429)

Spc

Nonlinear Regression **Equation:** f = a/(1+exp(-(x-x0)*b))

R	Rsqr	Adj Rsqr	Standard Error of Estimate
0.9791	0.9586	0.9380	0.2907

	Coefficient	Std. Error	t	P
a	8.8611	6.7855	1.3059	0.2616
b	0.2703	0.1356	1.9939	0.1169
x0	7.5251	5.6853	1.3236	0.2562

Statistical Tests:
Normality Test (Shapiro-Wilk) Passed (P = 0.2417)
W Statistic= 0.8833 Significance Level = 0.0500
Constant Variance Test Passed (P = 0.7200)

Table 7.3: Plasmid copy number

Strain	Plasmid size (bp)	Concentration of genomic DNA (μg/mL)						Concentration of plasmid DNA (μg/mL)						Plasmid copy number					
		6hrs	24 hrs	7 days	14 days	21 days	28 days	6hrs	24 hrs	7 days	14 days	21 days	28 days	6hrs	24 hrs	7 days	14 days	21 days	28 days
WT	0	27.59	112.61	119.97	105.71	107.34	107.69												
pME4510	4017	21.87	101.05	110.33	116.39	112.92	114.93	1.70	5.44	5.93	6.20	5.91	6.14	**121.8**	**84.5**	**84.3**	**83.5**	**82.1**	**83.8**
pME*lux*	9844	8.28	37.00	33.58	32.74	31.75	35.32	1.31	3.12	2.49	2.78	2.54	2.88	**101.0**	**53.9**	**47.4**	**54.4**	**51.1**	**52.2**
ldcC	9934	9.74	32.25	29.19	30.64	32.23	30.11	1.52	2.45	2.31	2.52	2.60	2.49	**99.1**	**48.2**	**50.3**	**52.1**	**51.2**	**52.3**
spc	9934	8.95	33.06	32.98	32.76	31.12	30.89	1.35	2.76	2.40	2.33	2.48	2.49	**95.6**	**52.9**	**46.2**	**45.1**	**50.5**	**51.1**
lys R25	9943	8.12	34.58	32.09	31.05	32.86	26.79	1.27	2.48	2.03	2.47	2.32	2.33	**99.3**	**45.5**	**40.1**	**50.5**	**44.7**	**55.2**
lys G25	9968	9.17	34.04	29.29	30.32	31.49	32.31	1.38	2.53	2.30	2.49	2.45	2.62	**95.5**	**47.0**	**49.6**	**51.9**	**49.2**	**51.2**
lpp R3	9968	9.35	38.80	27.72	30.07	32.78	31.72	1.40	2.52	1.93	2.24	2.55	2.43	**94.6**	**41.1**	**44.0**	**47.2**	**49.1**	**48.3**
lpp R4	10014	9.88	35.86	27.17	28.04	30.70	33.35	1.53	2.66	2.01	2.35	2.31	2.58	**97.6**	**46.7**	**46.5**	**52.7**	**47.4**	**48.6**
tat H5	10014	5.90	34.97	28.31	27.78	28.30	33.33	0.88	2.72	1.89	1.93	2.00	2.49	**93.7**	**48.9**	**42.0**	**43.8**	**44.4**	**47.0**
tat H9	10014	9.42	35.68	27.20	28.65	30.08	32.07	1.45	2.55	1.86	2.27	2.42	2.39	**96.8**	**44.9**	**42.9**	**49.9**	**50.7**	**46.9**
tat H14	10032	8.34	35.68	29.88	31.66	29.47	32.17	1.29	2.74	2.22	2.39	2.28	2.30	**97.0**	**48.3**	**46.6**	**47.4**	**48.7**	**44.9**

Size of genome in each case is 6300000bp.

Table 7.4: Relationship between time and plasmid copy number for all plasmid-bearing strains.

		N	Correlation	Sig.
Pair 1	Time & pME4510_PCN	6	-.539	.270
Pair 2	Time & pME*lux*_PCN	6	-.503	.309
Pair 3	Time & ldcC_PCN	6	-.453	.366
Pair 4	Time & spc_PCN	6	-.493	.321
Pair 5	Time & lysR25_PCN	6	-.354	.492
Pair 6	Time & lysG25_PCN	6	-.463	.356
Pair 7	Time & lppR3_PCN	6	-.391	.443
Pair 8	Time & lppR4_PCN	6	-.483	.332
Pair 9	Time & tatH5_PCN	6	-.515	.296
Pair 10	Time & tatH9_PCN	6	-.433	.391
Pair 11	Time & tatH14_PCN	6	-.532	.277

Appendix 9: Bacterial growth and gene expression

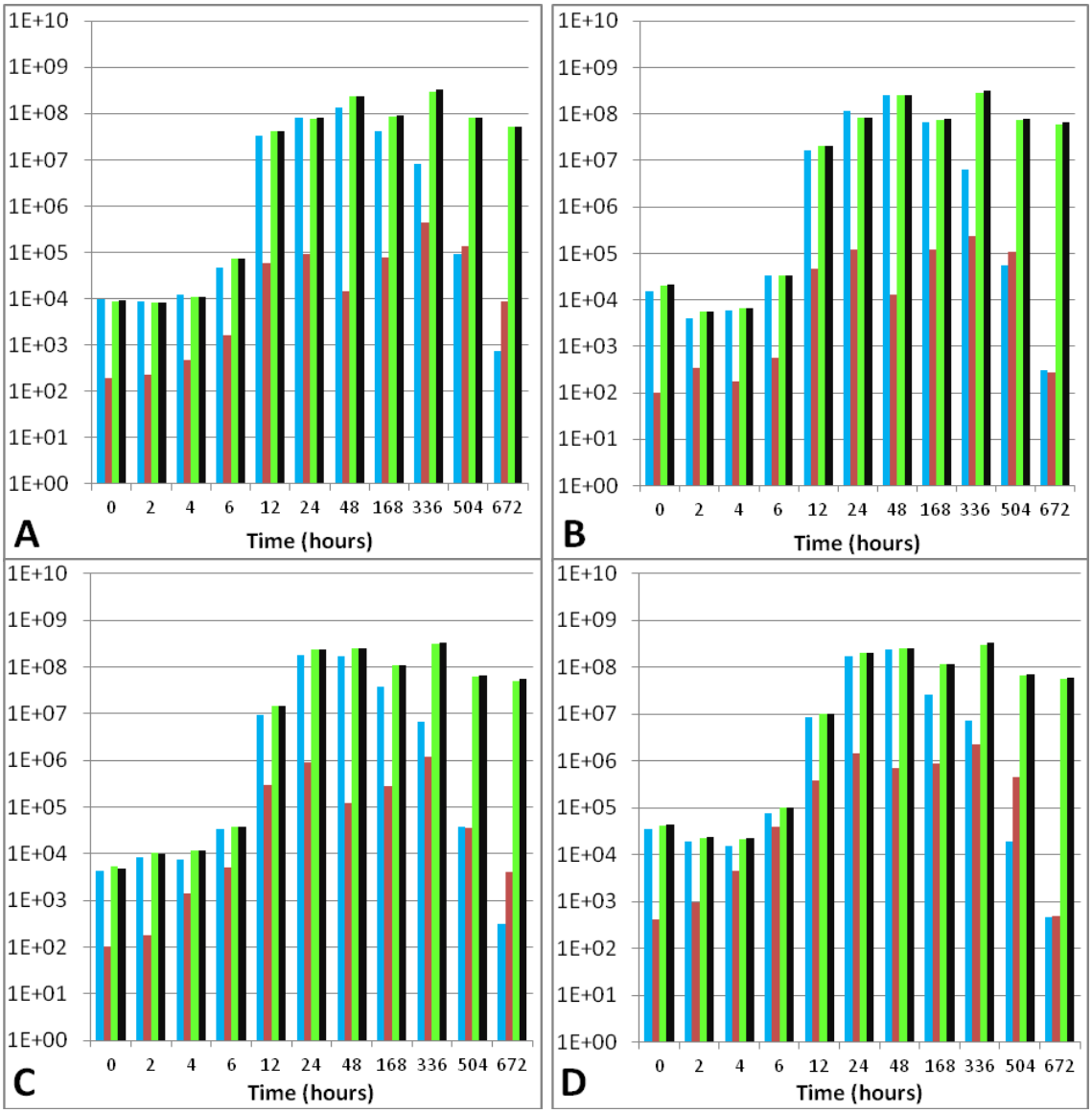


Figure 7.13: Comparisons of bioluminescence (RLU), colony forming units (CFU), live fluorescence (RFU) and total RFU at various time points. Time in hours is represented on X-axis of all graphs. CFU, RLU, Live RFU and Total RFU. A: ldcC, B: spc, C: tat, D: lpp.

Appendix 10: Preservative efficacy testing

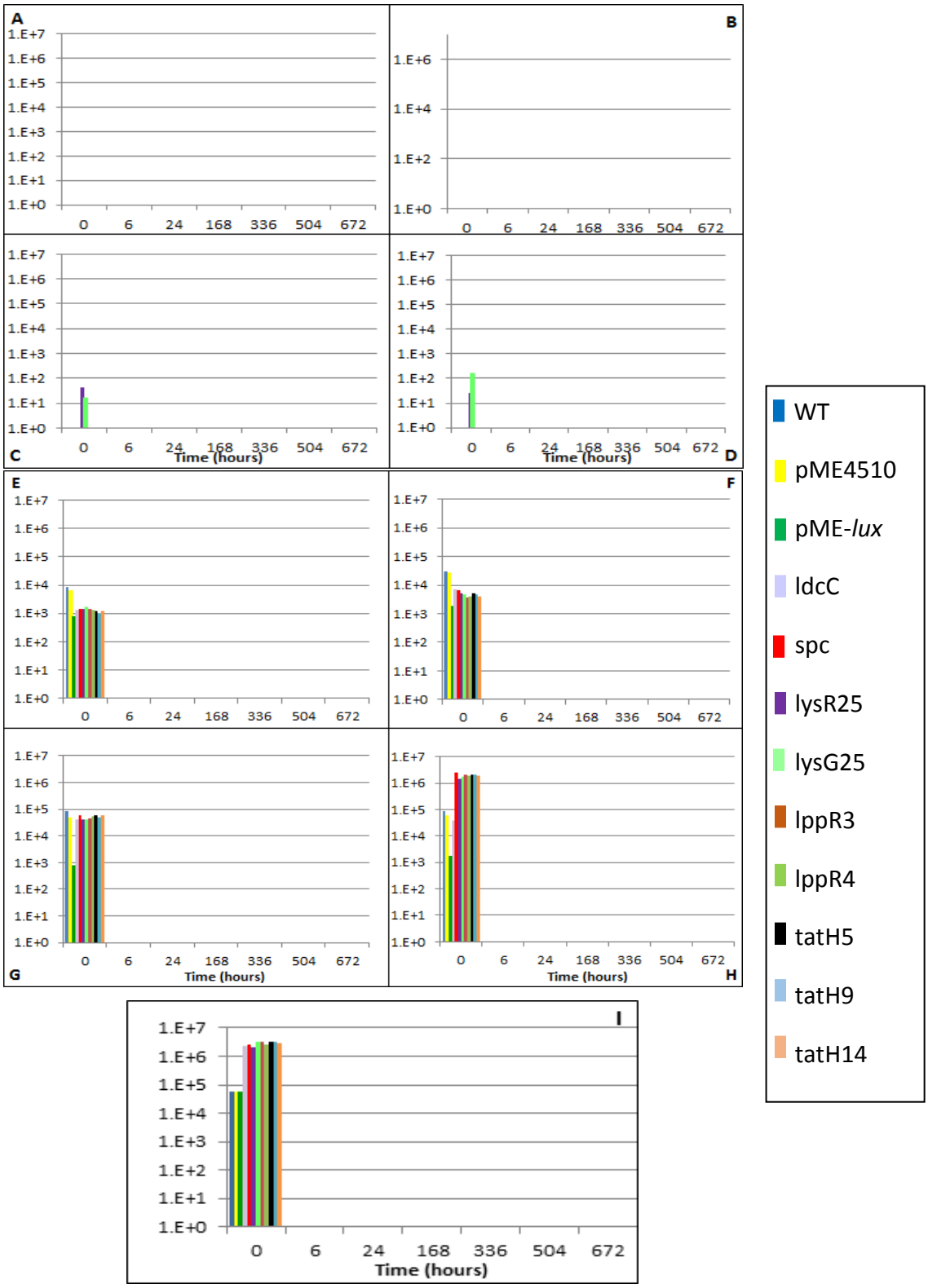


Figure 7.14: BKC efficacy test for all strains. Relationship between relative bioluminescence (A-D), colony forming units (E-H) and relative fluorescence (I) against time for all strains challenged with 0.02% (A, E), 0.01% (B, F), 0.005% (C, G) and 0.0025% (D, H, I) BKC.

Table 7.5: Correlations between RLU, CFU and RFU for all strains challenged with various concentrations of BKC.

	BKC Conc		WT	pME4510	pME*lux*	ldcC	spc	lysR25	lysG25	lppR3	lppR4	tatH5	tatH9	tatH14
CFU:RLU	0.02%	Correl	-.444	.431	.027	.949**	.156	.344	-.917**	-.110	.524	-.575	.753	-.300
		Sig	.318	.334	.955	.001	.738	.450	.004	.815	.228	.177	.051	.514
	0.01%	Correl	-.070	-.257	-.189	-.320	.525	.652	.534	.090	.187	-.087	-.083	.051
		Sig	.881	.578	.685	.484	.226	.113	.217	.847	.687	.854	.860	.914
	0.005%	Correl	.640	-.102	.467	-.025	-.093	.805*	.781*	.457	-.182	.632	.575	.691
		Sig	.122	.828	.291	.957	.842	.029	.038	.303	.696	.128	.177	.086
	0.0025%	Correl	-.045	.422	-.228	.549	.427	.907**	.945**	.906**	.901**	.959**	.901**	.957**
		Sig	.924	.346	.623	.202	.339	.005	.001	.005	.006	.001	.006	.001
	0.00125%	Correl	.221	.547	-.018	.247	-.011	.995**	.999**	.997**	.997**	.993**	.962**	.998**
		Sig	.633	.204	.969	.593	.981	.000	.000	.000	.000	.000	.001	.000
	0.0006%	Correl	-.577	-.582	-.306	.999**	.998**	1.000**	1.000**	.999**	1.000**	1.000**	1.000**	1.000**
		Sig	.175	.171	.504	.000	.000	.000	.000	.000	.000	.000	.000	.000
	0.0003%	Correl	.361	.395	.392	.985**	.989**	.962**	.990**	.998**	.986**	.968**	.992**	.997**
		Sig	.427	.380	.385	.000	.000	.001	.000	.000	.000	.000	.000	.000
	0.0000%	Correl	.072	.159	-.098	.401	.118	.368	.452	.387	.382	.635	.380	.485
		Sig	.878	.734	.835	.373	.801	.416	.309	.391	.398	.125	.401	.270
RLU:RFU	0.02%	Correl												
		Sig												
	0.01%	Correl												
		Sig												
	0.005%	Correl												
		Sig												
	0.0025%	Correl	-.077	.354	-.313	.550	.428	.908**	.945**	.906**	.902**	.959**	.901**	.957**
		Sig	.869	.436	.494	.201	.338	.005	.001	.005	.005	.001	.006	.001
	0.00125%	Correl	.316	.552	.033	.250	-.009	.995**	.999**	.997**	.998**	.994**	.962**	.998**
		Sig	.490	.199	.943	.589	.985	.000	.000	.000	.000	.000	.001	.000
	0.0006%	Correl	-.390	-.512	-.206	.976**	.967**	.864*	.943**	.933**	.933**	.976**	.974**	.926**
		Sig	.387	.240	.657	.000	.000	.012	.001	.002	.002	.000	.000	.003
	0.0003%	Correl	.351	.379	.401	.976**	.984**	.933**	.918**	.995**	.974**	.991**	.982**	.998**
		Sig	.439	.402	.372	.000	.000	.002	.004	.000	.000	.000	.000	.000
	0.0000%	Correl	.040	.215	-.221	-.042	-.035	-.114	.457	.083	-.090	.625	.399	-.179
		Sig	.932	.644	.633	.929	.940	.807	.303	.860	.847	.133	.376	.700
CFU:RFU	0.02%	Correl												
		Sig												
	0.01%	Correl												
		Sig												
	0.005%	Correl												
		Sig												
	0.0025%	Correl	.988**	.993**	.987**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
		Sig	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
	0.00125%	Correl	.989**	.996**	.986**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**	1.000**
		Sig	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
	0.0006%	Correl	.891**	.932**	.848*	.967**	.953**	.854*	.936**	.920**	.922**	.973**	.974**	.921**
		Sig	.007	.002	.016	.000	.001	.015	.002	.003	.003	.000	.000	.003
	0.0003%	Correl	.999**	.999**	.999**	.999**	.996**	.987**	.965**	.998**	.997**	.931**	.957**	.994**
		Sig	.000	.000	.000	.000	.000	.000	.000	.000	.000	.002	.001	.000
	0.0000%	Correl	.982**	.961**	.856*	.386	.934**	.514	.992**	.911**	.808*	.953**	.977**	.747
		Sig	.000	.001	.014	.393	.002	.238	.000	.004	.028	.001	.000	.053

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 7.6: Correlations between RLU, CFU and RFU for strains challenged with various concentrations of BA.

	BA Conc		WT	pME4510	pME*lux*	ldcC	spc	lysR25	lppR4	tatH5
CFU:RLU	2%	Correl				.798*	.815*	.950**	.818*	.793*
		Sig				.031	.025	.001	.025	.034
	1%	Correl				.955**	.923**	.988**	.981**	.989**
		Sig				.001	.003	.000	.000	.000
	0.5%	Correl				.992**	1.000**	.990**	.990**	.993**
		Sig				.000	.000	.000	.000	.000
	0.25%	Correl				.997**	.991**	.984**	.991**	.994**
		Sig				.000	.000	.000	.000	.000
	0.125%	Correl				.780*	.874*	.777*	.856*	.898**
		Sig				.039	.010	.040	.014	.006
	0.06%	Correl				.891**	.765*	.959**	.918**	.923**
		Sig				.007	.045	.001	.004	.003
RLU:RFU	2%	Correl				.799*	.818*	.952**	.826*	.798*
		Sig				.031	.025	.001	.022	.031
	1%	Correl				.960**	.931**	.989**	.995**	.996**
		Sig				.001	.002	.000	.000	.000
	0.5%	Correl				.998**	.999**	.990**	.997**	.997**
		Sig				.000	.000	.000	.000	.000
	0.25%	Correl				.985**	.978**	.985**	.992**	.994**
		Sig				.000	.000	.000	.000	.000
	0.125%	Correl				.770*	.896**	.804*	.826*	.905**
		Sig				.043	.006	.029	.022	.005
	0.06%	Correl								
		Sig								
CFU:RFU	2%	Correl	.948**	.998**	.998**	.999**	1.000**	1.000**	1.000**	1.000**
		Sig	.001	.000	.000	.000	.000	.000	.000	.000
	1%	Correl	.954**	.925**	.909**	.999**	.992**	.989**	.989**	.992**
		Sig	.001	.003	.005	.000	.000	.000	.000	.000
	0.5%	Correl	.998**	.976**	.998**	.998**	1.000**	.999**	.998**	.998**
		Sig	.000	.000	.000	.000	.000	.000	.000	.000
	0.25%	Correl	.982**	.975**	.985**	.991**	.995**	.998**	.999**	.998**
		Sig	.000	.000	.000	.000	.000	.000	.000	.000
	0.125%	Correl	.998**	.992**	.999**	1.000**	.999**	.998**	.998**	1.000**
		Sig	.000	.000	.000	.000	.000	.000	.000	.000
	0.06%	Correl								
		Sig								

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 7.7: Log unit reductions of RLU and CFU for all strains at various BKC concentrations

0.02% BKC														
	CFU	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**
	0	**6**	**24**	**168**	**336**	**504**	**672**	**0**	**6**	**24**	**168**	**336**	**504**	**672**
WT	2.59	6.51	-	-	-	-	-							
pME4510	2.29	6.11	-	-	-	-	-							
pME*lux*	3.57	6.48	-	-	-	-	-							
ldcC	3.49	6.60	-	-	-	-	-	3.65	-	-	-	-	-	-
spc	3.39	6.57	-	-	-	-	-	3.68	-	-	-	-	-	-
lys R25	3.28	6.42	-	-	-	-	-	4.57	-	-	-	-	-	-
lys G25	3.24	6.47	-	-	-	-	-	4.49	-	-	-	-	-	-
lpp R3	3.41	6.56	-	-	-	-	-	4.34	-	-	-	-	-	-
lpp R4	3.36	6.48	-	-	-	-	-	4.42	-	-	-	-	-	-
tat H5	3.34	6.42	-	-	-	-	-	4.27	-	-	-	-	-	-
tat H9	3.40	6.40	-	-	-	-	-	4.43	-	-	-	-	-	-
tat H14	3.32	6.40	-	-	-	-	-	4.38	-	-	-	-	-	-

0.01% BKC														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	2.03	6.51	-	-	-	-	-							
pME4510	1.67	6.11	-	-	-	-	-							
pME*lux*	3.22	6.48	-	-	-	-	-							
ldcC	2.75	6.60	-	-	-	-	-	3.65	-	-	-	-	-	-
spc	2.76	6.57	-	-	-	-	-	3.68	-	-	-	-	-	-
lys R25	2.72	6.42	-	-	-	-	-	4.57	-	-	-	-	-	-
lys G25	2.79	6.47	-	-	-	-	-	4.49	-	-	-	-	-	-
lpp R3	3.00	6.56	-	-	-	-	-	4.34	-	-	-	-	-	-
lpp R4	2.86	6.48	-	-	-	-	-	4.42	-	-	-	-	-	-
tat H5	2.71	6.42	-	-	-	-	-	4.27	-	-	-	-	-	-
tat H9	2.74	6.40	-	-	-	-	-	4.43	-	-	-	-	-	-
tat H14	2.78	6.40	-	-	-	-	-	4.38	-	-	-	-	-	-

0.005% BKC														
	CFU	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**
	0	**6**	**24**	**168**	**336**	**504**	**672**	**0**	**6**	**24**	**168**	**336**	**504**	**672**
WT	1.60	6.51	-	-	-	-	-							
pME4510	1.43	6.11	-	-	-	-	-							
pME*lux*	3.57	6.48	-	-	-	-	-							
ldcC	1.99	6.60	-	-	-	-	-	3.65	-	-	-	-	-	-
spc	1.79	6.57	-	-	-	-	-	3.68	-	-	-	-	-	-
lys R25	1.79	6.42	-	-	-	-	-	2.96	-	-	-	-	-	-
lys G25	1.84	6.47	-	-	-	-	-	3.29	-	-	-	-	-	-

lpp R3	1.88	6.56	-	-	-	-	-	4.34	-	-	-	-	-	-
lpp R4	1.76	6.48	-	-	-	-	-	4.42	-	-	-	-	-	-
tat H5	1.67	6.42	-	-	-	-	-	4.27	-	-	-	-	-	-
tat H9	1.71	6.40	-	-	-	-	-	4.43	-	-	-	-	-	-
tat H14	1.64	6.40	-	-	-	-	-	4.38	-	-	-	-	-	-

0.0025% BKC														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672							
WT	1.55	6.51	-	-	-	-	-							
pME4510	1.31	6.11	-	-	-	-	-							
pME*lux*	3.22	6.48	-	-	-	-	-							
ldcC	0.46	6.60	-	-	-	-	-	3.65	-	-	-	-	-	-
spc	0.18	6.57	-	-	-	-	-	3.68	-	-	-	-	-	-
lys R25	0.26	6.42	-	-	-	-	-	2.28	4.57	-	-	-	-	-
lys G25	0.23	6.47	-	-	-	-	-	2.28	4.49	-	-	-	-	-
lpp R3	0.23	6.56	-	-	-	-	-	4.34	4.34	-	-	-	-	-
lpp R4	0.18	6.48	-	-	-	-	-	2.48	4.42	-	-	-	-	-
tat H5	0.08	6.42	-	-	-	-	-	4.27	4.27	-	-	-	-	-
tat H9	0.09	6.40	-	-	-	-	-	4.43	4.43	-	-	-	-	-
tat H14	0.10	6.40	-	-	-	-	-	4.38	4.38	-	-	-	-	-

0.00125% BKC														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	1.51	6.51	-	-	-	-	-							
pME4510	1.24	6.11	-	-	-	-	-							
pME*lux*	2.73	6.48	-	-	-	-	-							
ldcC	0.24	6.60	-	-	-	-	-	3.65	-	-	-	-	-	-
spc	0.16	6.57	-	-	-	-	-	3.68	-	-	-	-	-	-
lys R25	0.25	6.42	-	-	-	-	-	1.56	4.57	-	-	-	-	-
lys G25	0.13	6.47	-	-	-	-	-	1.17	4.49	-	-	-	-	-
lpp R3	0.22	6.56	-	-	-	-	-	1.29	4.34	-	-	-	-	-
lpp R4	0.14	6.48	-	-	-	-	-	1.30	4.42	-	-	-	-	-
tat H5	-0.02	6.42	-	-	-	-	-	1.70	4.27	-	-	-	-	-
tat H9	0.04	6.40	-	-	-	-	-	1.72	4.43	-	-	-	-	-
tat H14	0.03	6.40	-	-	-	-	-	2.19	4.38	-	-	-	-	-

0.000625% BKC														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672							
WT	1.21	6.51	-	-	-	-	-							
pME4510	0.78	6.11	-	-	-	-	-							
pME*lux*	2.68	6.48	-	-	-	-	-							

ldcC	0.24	2.32	3.30	6.60	-	-	-	0.45	3.65	-	-	-	-	-
spc	0.13	2.29	3.03	6.57	-	-	-	0.78	3.68	-	-	-	-	-
lys R25	0.04	2.35	3.06	6.42	-	-	-	0.88	2.83	4.57	-	-	-	-
lys G25	0.06	2.30	2.83	6.47	-	-	-	0.85	2.88	4.49	-	-	-	-
lpp R3	0.11	2.48	3.41	6.56	-	-	-	0.67	2.36	4.34	-	-	-	-
lpp R4	0.11	2.34	3.11	6.48	-	-	-	0.78	2.51	4.42	-	-	-	-
tat H5	-0.04	2.18	3.02	6.42	-	-	-	0.53	4.27	4.27	-	-	-	-
tat H9	0.02	2.29	2.96	6.40	-	-	-	0.73	4.43	4.43	-	-	-	-
tat H14	0.00	2.31	2.89	6.40	-	-	-	0.90	4.38	4.38	-	-	-	-

0.0003125% BKC														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	0.93	6.51	-	-	-	-	-							
pME4510	0.76	6.11	-	-	-	-	-							
pME*lux*	0.90	6.48	-	-	-	-	-							
ldcC	0.16	1.39	1.54	3.59	6.60	-	-	0.16	0.82	3.65	-	-	-	-
spc	0.10	0.82	1.14	3.09	6.57	-	-	0.28	0.85	3.68	-	-	-	-
lys R25	0.03	0.39	0.87	2.76	3.72	-	-	0.64	1.51	2.66	4.57	-	-	-
lys G25	0.01	0.36	1.41	2.99	6.47	-	-	0.77	1.48	2.56	4.49	-	-	-
lpp R3	0.06	0.64	1.35	3.48	6.56	-	-	0.55	1.13	3.39	4.34	-	-	-
lpp R4	0.03	0.40	1.11	3.12	6.48	-	-	0.54	1.28	2.36	4.42	-	-	-
tat H5	-0.06	0.70	1.21	3.20	6.42	-	-	0.31	1.35	2.50	4.27	-	-	-
tat H9	-0.01	0.24	0.71	2.83	3.86	-	-	0.64	1.46	2.36	4.43	-	-	-
tat H14	0.01	0.52	0.91	3.36	3.77	-	-	0.69	1.38	2.88	4.38	-	-	-

0% BKC														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	**6**	**24**	**168**	**336**	**504**	**672**	**0**	**6**	**24**	**168**	**336**	**504**	**672**
WT	0.00	-0.13	-0.24	-0.19	-0.19	-0.10	-0.18							
pME4510	0.00	-0.46	-0.54	-0.52	-0.62	-0.68	-0.63							
pME*lux*	0.00	-0.10	0.15	0.16	-0.08	-0.07	0.00							
ldcC	0.00	0.50	0.56	0.43	0.57	0.55	0.27	0.00	0.03	0.06	0.69	0.40	0.88	0.59
spc	0.00	0.24	0.45	0.25	0.51	0.46	0.00	0.00	0.03	0.05	0.43	0.58	0.73	0.96
lys R25	0.00	0.27	0.31	0.26	0.48	0.16	0.24	0.00	0.01	0.02	0.61	0.65	0.78	0.64
lys G25	0.00	0.18	0.47	0.38	0.36	0.12	0.35	0.00	0.03	0.09	0.50	0.44	0.50	0.40
lpp R3	0.00	0.57	0.83	0.78	0.44	0.48	0.26	0.00	0.07	0.09	0.35	0.43	0.54	0.36
lpp R4	0.00	0.29	0.48	0.48	0.08	0.47	0.31	0.00	0.03	0.07	0.33	0.27	0.28	0.18
tat H5	0.00	0.03	0.26	0.60	-0.02	0.28	0.34	0.00	0.04	0.07	0.46	0.41	0.50	0.29
tat H9	0.00	0.27	0.39	0.67	0.40	0.21	0.25	0.00	0.07	0.11	0.43	0.41	0.41	0.29
tat H14	0.00	0.38	0.81	0.74	0.40	0.35	0.33	0.00	0.02	0.04	0.37	0.38	0.43	0.28

Table 7.8: Log unit reductions of RLU and CFU for all strains at various BA concentrations.

2% BA														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	2.04	6.51	-	-	-	-	-							
pME4510	2.88	6.11	-	-	-	-	-							
pME*lux*	3.24	6.32	-	-	-	-	-							
ldcC	0.21	6.60	-	-	-	-	-	1.25	3.65	-	-	-	-	-
spc	0.14	6.57	-	-	-	-	-	1.26	3.68	-	-	-	-	-
lys R25	0.03	6.42	-	-	-	-	-	1.70	4.57	-	-	-	-	-
lpp R4	0.13	6.48	-	-	-	-	-	1.86	4.42	-	-	-	-	-
tat H5	0.08	6.42	-	-	-	-	-	1.99	4.27	-	-	-	-	-

1% BA														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	0.27	6.51	-	-	-	-	-							
pME4510	0.03	6.11	-	-	-	-	-							
pME*lux*	0.19	6.32	-	-	-	-	-							
ldcC	0.18	0.49	0.87	6.60	-	-	-	0.61	1.35	3.65	-	-	-	-
spc	0.13	0.57	0.86	6.57	-	-	-	0.85	3.68	3.68	-	-	-	-
lys R25	-0.03	0.38	0.68	6.42	-	-	-	1.07	1.67	2.30	4.57	-	-	-
lpp R4	0.05	0.44	0.72	6.48	-	-	-	0.90	1.48	2.42	4.42	-	-	-
tat H5	-0.02	0.36	0.65	6.42	-	-	-	0.79	1.28	1.97	4.27	-	-	-

0.5% BA														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	0.30	0.85	1.48	6.51	-	-	-							
pME4510	0.11	0.36	0.56	6.11	-	-	-							
pME*lux*	0.25	0.61	1.01	6.32	-	-	-							
ldcC	0.13	0.71	1.37	6.60	-	-	-	0.26	0.67	1.50	2.00	2.81	2.88	3.18
spc	0.10	0.68	1.35	6.57	-	-	-	0.32	0.93	1.54	2.15	3.21	2.98	3.38
lys R25	-0.10	0.53	1.21	6.42	-	-	-	0.33	0.79	1.09	2.28	2.28	2.30	2.28
lpp R4	-0.03	0.57	1.19	6.48	-	-	-	0.26	0.67	1.22	2.40	2.27	2.25	2.35
tat H5	-0.05	0.53	1.24	6.42	-	-	-	0.04	0.48	0.93	2.01	2.03	2.10	2.19

0.25% BA														
	CFU	CFU	CFU	CFU	CFU	CFU	CFU	RLU	RLU	RLU	RLU	RLU	RLU	RLU
	0	6	24	168	336	504	672	0	6	24	168	336	504	672
WT	0.49	0.53	0.76	0.86	2.37	2.39	2.41							
pME4510	0.11	0.12	0.35	0.41	1.29	1.29	1.26							
pME*lux*	0.18	0.31	0.50	0.71	1.40	1.45	1.51							

ldcC	0.13	0.53	1.20	1.56	1.83	1.79	1.72	-0.12	0.31	0.82	0.86	1.02	0.93	0.95
spc	0.10	0.40	1.22	1.39	1.76	1.84	1.67	0.09	0.46	1.04	1.04	1.33	1.16	1.01
lys R25	-0.11	0.38	1.01	1.34	1.51	1.51	1.47	0.06	0.52	0.64	0.64	0.78	0.67	0.68
lpp R4	-0.01	0.46	1.04	1.30	1.59	1.53	1.42	-0.10	0.37	0.60	0.60	0.79	0.67	0.67
tat H5	-0.05	0.30	0.99	1.30	1.48	1.63	1.61	-0.29	0.01	0.33	0.31	0.47	0.39	0.55

0.125% BA														
	CFU	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**
	0	**6**	**24**	**168**	**336**	**504**	**672**	**0**	**6**	**24**	**168**	**336**	**504**	**672**
WT	0.25	0.48	0.49	1.33	1.27	1.13	1.00							
pME4510	-0.15	-0.07	0.03	1.16	0.96	0.99	1.03							
pME*lux*	0.13	0.27	0.29	1.46	1.37	1.35	1.36							
ldcC	0.16	0.25	0.29	1.95	2.01	2.03	1.83	-0.04	0.33	1.08	1.24	1.20	1.13	1.32
spc	0.14	0.17	0.38	1.86	2.01	2.25	1.86	0.21	0.45	1.94	2.22	2.24	2.15	2.21
lys R25	-0.12	-0.05	0.01	1.53	1.81	1.96	1.55	0.02	0.38	0.77	0.79	0.93	0.91	1.01
lpp R4	-0.05	0.07	0.17	1.89	1.96	1.93	1.68	0.08	0.41	0.71	0.70	0.92	0.92	1.03
tat H5	-0.13	0.00	0.14	1.76	1.81	1.79	1.58	-0.11	0.13	0.55	0.54	0.71	0.74	0.89

0.0625% BA														
	CFU	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**
	0	**6**	**24**	**168**	**336**	**504**	**672**	**0**	**6**	**24**	**168**	**336**	**504**	**672**
WT	0.51	0.46	0.42	0.27	0.22	0.10	0.09							
pME4510	-0.07	-0.15	-0.19	0.10	0.09	-0.17	-0.19							
pME*lux*	0.15	0.18	-0.26	-0.25	0.31	0.22	-0.12							
ldcC	0.13	0.15	0.03	-0.05	0.14	0.11	0.06	-0.19	-0.08	-0.29	-0.31	-0.13	-0.13	-0.17
spc	0.20	0.18	-0.04	-0.06	0.25	0.17	0.04	0.00	0.05	-0.13	-0.16	-0.09	-0.08	-0.10
lys R25	0.10	0.01	-0.25	-0.26	-0.05	-0.11	-0.08	-0.02	0.02	-0.17	-0.19	-0.05	-0.07	-0.08
lpp R4	-0.08	0.06	-0.23	-0.19	-0.01	-0.03	-0.09	0.02	0.13	-0.14	-0.08	-0.02	0.02	0.03
tat H5	0.00	0.09	-0.20	-0.24	0.00	-0.05	-0.13	-0.16	-0.14	-0.25	-0.28	-0.18	-0.16	-0.27

0% BA														
	CFU	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**CFU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**	**RLU**
	0	**6**	**24**	**168**	**336**	**504**	**672**	**0**	**6**	**24**	**168**	**336**	**504**	**672**
WT	0	-0.13	-0.24	-0.19	-0.19	-0.10	-0.18							
pME4510	0	-0.46	-0.54	-0.52	-0.62	-0.68	-0.63							
pME*lux*	0	0.00	-0.01	0.00	-0.23	-0.22	-0.15							
ldcC	0	0.50	0.56	0.43	0.57	0.55	0.27	0.00	0.03	0.06	0.69	0.40	0.88	0.59
spc	0	0.24	0.45	0.25	0.51	0.46	0.00	0.00	0.03	0.05	0.43	0.58	0.73	0.96
lys R25	0	0.27	0.31	0.26	0.48	0.16	0.24	0.00	0.01	0.02	0.61	0.65	0.78	0.64
lpp R4	0	0.29	0.48	0.48	0.08	0.47	0.31	0.00	0.03	0.07	0.33	0.27	0.28	0.18
tat H5	0	0.03	0.26	0.60	-0.02	0.28	0.34	0.00	0.04	0.07	0.46	0.41	0.50	0.29

Table 7.9: Equivalence between bioluminescence and plate count method.

	Significance values for BKC concentrations								Significance values for BA concentrations					
Strain	**0**	**0.02%**	**0.01%**	**0.005%**	**0.0025%**	**0.00125%**	**0.0006%**	**0.0003%**	**2%**	**1%**	**0.50%**	**0.25%**	**0.13%**	**0.06%**
ldcC	0.000	0.277	0.419	0.447	0.454	0.454	0.447	0.379	0.454	0.128	0.250	0.142	0.040	0.000
spc	0.000	0.291	0.424	0.449	0.454	0.454	0.448	0.265	0.454	0.155	0.250	0.121	0.047	0.000
lys R25	0.000	0.408	0.403	0.450	0.454	0.455	0.451	0.158	0.454	0.149	0.270	0.168	0.041	0.000
lys G25	0.000	0.363	0.426	0.448	0.454	0.455	0.45	0.199	nd	nd	nd	nd	nd	nd
lpp R3	0.001	0.293	0.389	0.447	0.454	0.455	0.452	0.254	nd	nd	nd	nd	nd	nd
lpp R4	0.000	0.283	0.379	0.448	0.454	0.455	0.45	0.185	0.454	0.140	0.257	0.145	0.048	0.000
tat H5	0.000	0.331	0.387	0.447	0.454	0.454	0.45	0.298	0.454	0.137	0.260	0.133	0.051	0.000
tat H9	0.000	0.334	0.384	0.446	0.454	0.454	0.45	0.122	nd	nd	nd	nd	nd	nd
tat H14	0.000	0.341	0.372	0.449	0.454	0.454	0.45	0.2	nd	nd	nd	nd	nd	nd

ANOVA (Significance level P<0.05). Values greater than 0.05 indicate no significant difference between both methods (highlighted in green). nd – not determined

Appendix 11: List of posters and presentations

Shah, N. & Naseby, D. (2013). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Poster for 11th LFS Annual Research Day University of Hertfordshire 2013. 15 May 2013.

Shah, N. & Naseby, D. (2012). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Poster for *ISME14 – The power of the Small*. International Symposium of Microbial Ecology, Copenhagen, Denmark. 19-24 August 2012.

Shah, N. & Naseby, D. (2012). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Poster for 10th LFS Annual Research Day. University of Hertfordshire 2012.

Shah, N. & Naseby, D. (2012). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Presentation at Postgraduate club, University of Hertfordshire.

Shah, N. & Naseby, D. (2012). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Poster for Society of General Microbiology Spring Conference 2012. Convention Centre Dublin, Ireland. 26-29 March 2012.

Shah, N. & Naseby, D. (2011). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Poster for Society of General Microbiology Autumn Conference 2011. University of York, UK. 5-7 September 2011.

Shah, N. & Naseby, D. (2011). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing. Poster for 9th LFS Annual Research Day, University of Hertfordshire 2011. 4 May 2011.

Shah, N. & Naseby, D. (2011). Development of *lux*-based biosensors in *Pseudomonas aeruginosa* ATCC 9027 for preservative efficacy testing**.** Presentation at Postgraduate club, University of Hertfordshire.