Kingston University London

Azoreductases: genes and proteins in *Pseudomonas aeruginosa*

A thesis submitted in partial fulfilment for the degree of

Doctor of Philosophy

By

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September 2015

Declaration

The thesis entitled *Azoreductases: genes and proteins in Pseudomonas aeruginosa* is based upon the work conducted in the Faculty of Science, Engineering and Computing at Kingston University London and in collaboration with Dr. Gail Preston's group in the Plant Sciences Department at the University of Oxford. All of the work described here is the candidatre's own original work unless otherwise acknowledged in the text or by references. None of the work presented here has been submitted for another degree in this or any other university.

Acknowledgments

I would like to express my gratitude to my supervisor, Prof. Edith Sim, whose expertise, understanding, and patience, added considerably to my PhD experience. I appreciate her vast knowledge and skill in many areas and her assistance in writing reports, presentations and this thesis. I consider the research experience that I gained under her supervision of inestimable value. A special thanks goes to Dr. Ali Ryan for the daily laboratory supervision and the support given at all levels of the research project, including the writing up of this thesis. I also would like to thank Dr. Gail Preston and Prof. Mark Fielder for the assistance they provided during this research project.

I must also acknowledge Sinead Holland, Dr. Elena Polycarpou, Olga Eleftheriadou and Dr. Mike Longman, my lab and office group. Their help, support and friendship have been really important every day during this experience.

A big thank you goes to my family and friends for the support they provided me through my entire life and in particular, I must acknowledge my wife, Alessandra, without whose love and encouragement, I would not have finished this thesis.

Publications

Azoreductases: novel determinants of fluoroquinolone resistance.
Crescente V, Holland SM, Cullen L, Polycarpou E, Preston GM, Sim E and Ryan A.
(Manuscript in preparation).

Identification of novel members of the bacterial azoreductase family in *Pseudomonas aeruginosa*.
Vincenzo Crescente, Sinead M. Holland, Sapna Kashyap, Elena Polycarpou, Edith Sim, Ali Ryan
Biochemical Journal Mar 2016, 473 (5) 549-558; DOI: 10.1042/BJ20150856.
(Copy attached).

Identification of NAD(P)H Quinone Oxidoreductase Activity in Azoreductases from *P. aeruginosa*: Azoreductases and NAD(P)H Quinone Oxidoreductases Belong to the Same FMN-Dependent Superfamily of Enzymes.
Ryan A, Kaplan E, Nebel J-C, Polycarpou E, Crescente V, Lowe E, Preston GM and Sim E (2014).
PLoS ONE 9(6): e98551. DOI:10.1371/journal.pone.0098551.
(Copy attached).

Presentations

Azoreductases: molecular and physiological roles in *Pseudomonas aeruginosa*.
Oral presentation, Research and Enterprise Development Day, Kingston Universty London,
May 2014.

Azoreductase: novel determinats of antibiotic sensitivity.
Poster presentation, Society for Applied Microbiology, autumn meeting, October 2013.

Azoreductase: novel determinats of antibiotic sensitivity.
Poster presentation, 14th international Conference on Pseudomonas, September 2013.

Azoreductase: novel determinats of antibiotic sensitivity.
Poster presentation, Interdisciplinary Hub Conference for the Study of Health and Age-related conditions (IhSHA), Kingston Universty London, July 2013.

Azoreductase: a potential novel target for antimicrobial agents.
Oral presentation, School of Life Sciences Research Day, Kingston University London, June 2012.

Table of contents

List of figures

List of tables

List of abbreviation

CF: cystic fibrosis
EPS: exopolysaccharide
VAP: ventilator-associated pneumonia
MDR: multi-drug resistant
ASL: airway surface liquid
CFTR: cystic fibrosis transmembrane regulator
MIC: minimal inhibitory concentration
MBC: minimal bactericidal concentration
LPS: lipopolysaccharide
QS: quorum sensing
IBD: inflammatory bowel disease
5-ASA: 5-aminosalicylic acid
FMN: flavin mononucleotide
FAD: flavin adenine dinucleotide
NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NQO: NAD(P)H quinone oxidoreductase
LB: Luria Bertani
BHI: brain heart infusion
PCR: polymerase chain reaction
TBE: tris borate EDTA
Rev: Reverse
Fwd: Forward
Kb: kilobase
bp: base pair
rpm: rotation per minute
OD: optical density
IPTG: isopropyl-β-D-thiogalactopyranoside
IMAC: immobilised metal-ion affinity chromatography
TLC: thin layer chromatography
R_f: retention factor
CV: crystal violet
NTA: nitrolotriacetic acid

Abstract

Pseudomonas aeruginosa is one of the primary causes of opportunistic infections in humans and it is associated with both acute and chronic infections in immunocompromised individuals. This bacterium is extremely resistant to many antibiotics, making the treatments against this pathogen often unsuccessful. Azoreductases, a family of enzymes involved in the reduction of azo compounds and quinones, are found in many bacterial species including *P. aeruginosa*. Although the enzymatic activity of three of these enzymes has been extensively characterized, their physiological role remains unclear. In this study, the enzymatic activity as well as the effect on physiological processes such as swarming motility, biofilm formation and antibiotic resistance of known and putative azoreductase proteins from *P. aeruginosa* PAO1 have been investigated. Five putative azoreductase genes from *P. aeruginosa* PAO1 (*pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*) were cloned and four of these (*pa0949*, *pa1204*, *pa2280* and *pa2580*) were over expressed in *E. coli* strains. Recombinant proteins were purified and biochemically characterized showing the presence of FAD bound to PA1204 and PA2580 proteins. Enzymatic reaction conditions were established for each protein by determining the preferred cofactor and reductant (flavin and NAD(P)H) used by each protein. Higher reduction rates were obtained using FAD for PA1204 and PA2580, and FMN for PA0949 and PA2280, whereas NADPH was always the preferred reductant for all of the enzymes tested. Substrate specificity studies performed with azo compounds and quinones showed that PA1204, PA2280 and PA2580 recombinant proteins can reduce both classes of substrate, with higher reduction rates with quinones, whereas the recombinant PA0949 protein showed to reduce only quinone substrates. Investigation of the role of azoreductase genes on *P. aeruginosa* PAO1 motility, biofilm formation and antibiotic resistance was conducted using single gene transposon mutants for each of the genes *paazor1*, *paazor2*, *paazor3*, *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*. Motility analysis demonstrated greater swarming for all mutants tested compared with wild type, although both wild type and mutants showed the same growth rate. Similarly, all mutants showed higher biofilm production compared with the wild type in a short term period (24 hours), whereas no differences were observed after a longer biofilm production period (48 hours). The MICs and MBCs of several antibiotics were determined, showing that, in presence of fluoroquinolones, *P. aeruginosa* PAO1 azoreductase mutants exhibit higher growth inhibition (up to 127-fold) and reduced survival (up to 7 fold) compared with wild type.

This suggests that azoreductase genes (or gene products) may be involved in the *P. aeruginosa* PAO1 resistance to antibiotic treatments, and in particular to fluoroquinolones. The findings prove that the proteins PA0949, PA1204, PA2280 and PA2580 have similar features and enzymatic functions to the already characterized paAzoR1-3 from *P. aeruginosa* PAO1. Therefore, these can be included in the family of azo- and quinone-oxidoreductase enzymes. The data presented here on the antibiotic resistance strongly suggest a role for azoreductase gene products in antimicrobial resistance in *P. aeruginosa*. These original findings provide a springboard for further investigation of azoreductases as novel targets for antimicrobial agents for this pathogen.

Chapter 1. Introduction

1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a member of the Gammaproteobacteria class, together with many important human pathogens such as Enterobacter, Salmonella, Yersinia and Escherichia. In 1984 *P. aeruginosa* was classified into the Pseudomonas genus which included polarly flagellated strictly aerobic rod bacteria (Palleroni, 1984). Although this bacterium is classified as aerobic, it has been demonstrated that *P. aeruginosa* is able to grow anaerobically using nitrate as the electron acceptor by fermentation of arginine, which makes this bacterium able to proliferate in conditions of partial or total oxygen depletion (Vander Wauven et al., 1984, Palmer et al., 2007). The adaptation to microaerobic or anaerobic environments is an essential factor for some *P. aeruginosa* lifestyles such as during lung infection in Cystic Fibrosis (CF) patients, where a thick layer of mucus and alginate surrounding bacterial cells can limit the oxygen diffusion (Williams et al., 2006, Hassett, 1996, Worlitzsch et al., 2002, Cooper et al., 2003).

As with many bacterial species, *P. aeruginosa* can proliferate in a planktonic form or biofilm. During planktonic growth, *P. aeruginosa* exists as single cells suspended in liquid, whereas a biofilm represents a type of bacterial proliferation which forms surface-associated microbial communities surrounded by an extracellular matrix composed primarily of bacterial-derived exopolysaccharides (EPS) (Branda et al., 2005, Hall-Stoodley and Stoodley, 2009, López et al., 2010).

The versatility of *P. aeruginosa* allows this bacterium to colonize a wide range of environmental habitats, including aquatic sediments, water exposed surfaces, soil, plant roots and leaves, human and animal sewage and it is a primary agent of opportunistic infection in humans (Ringen and Drake, 1952, Green et al., 1974b, Pellett et al., 1983c, Driscoll et al., 2007). The wide environmental adaptability of *P. aeruginosa* is also

reflected on the number of different hosts that can be infected by this pathogen such as humans, *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans* (roundworm), *Galleria mellonella* (greater wax moth), *Arabidopsis thaliana* (thale cress) and *Lactuca sativa* (lettuce) (Miyata et al., 2003, D'Argenio et al., 2001, Mahajan-Miklos et al., 1999, Rahme et al., 1997, Rahme et al., 1995, Apidianakis and Rahme, 2009, Tan et al., 1999, Starkey and Rahme, 2009b, Walker et al., 2004). In humans, *P. aeruginosa* is associated with both acute and chronic infections in immunocompromised individuals, and it is capable of infecting a multitude of sites and indwelling medical devices (Driscoll et al., 2007).

The ability of *P. aeruginosa* to adapt to a different set of environmental conditions and to cause distinct infections is also due to its ability to control gene expression in response to environmental stimuli. In fact, a substantial proportion of the *P. aeruginosa* genome is involved with regulatory systems that sense environmental conditions and integrate information to alter gene expression (Stover et al., 2000).

1.2. *P. aeruginosa* infections

P. aeruginosa is one of the major causes of life-threatening secondary infections in medical care facilities, responsible for more than 8% of all hospital-acquired infections (Sievert et al., 2013). Its genetic background allows *P. aeruginosa* to colonize and survive in many different environments, resulting in an organism that is phenotypically unstable, difficult to track epidemiologically, capable of persisting in different physical settings and highly capable in developing single, and more importantly, multi-drug resistance (Van Delden and Iglewski, 1998). Many epidemiological studies have compared environmental and clinical isolates of *P. aeruginosa*, showing that clinical isolates from acute infections are indistinguishable from environmental isolates (Nicas and Iglewski, 1986, Römling et al., 1994, Ferguson et al., 2001, Wolfgang et al., 2003, Pirnay et al., 2009). This suggests

that environmental strains are the primary source of *P. aeruginosa* infection, and the virulence characteristics of *P. aeruginosa* are pre-existing in the environment. These studies indicate that the environmental lifestyle of *P. aeruginosa* involves a repertoire of phenotypes that support and contribute to opportunistic human infections, making this pathogen one of the primary causes of opportunistic infections in humans (Driscoll et al., 2007).

In humans, *P. aeruginosa* is associated with both acute and chronic infections in immunocompromised patients suffering from AIDS, CF, cancer and pneumonia with the ability of infecting a multitude of sites and tissues, including lungs, burns, wounds, eyes and ears (Stover et al., 2000, Driscoll et al., 2007). One of the most recurring problems is the ability of *P. aeruginosa* to survive on medical devices (Driscoll et al., 2007). As mechanical ventilation is a necessity for patients with respiratory difficulties, hyperoxia (95% O_2 exposure) through mechanical ventilation increases the occurrence of *P. aeruginosa* infections (Lynch, 2001, Kremer et al., 2008) and *P. aeruginosa* is considered the causal agent for most ventilator associated pneumonias following mechanical ventilation (Rello et al., 2002, Fujitani et al., 2011). In particular, *P. aeruginosa* has become the principal species colonizing CF patients and the primary cause of morbidity and mortality due not only to the primary disease of the patient but also because of mechanical ventilation, an almost universal need for these patients (Murray et al., 2007, Chastre and Fagon, 2002, Flanagan et al., 2007).

1.2.1. Human infections

As mentioned above, the adaptability of *P. aeruginosa* and the high incidence of antibiotic resistance allow this pathogen to survive in many environments and under stressed conditions. As a consequence, *P. aeruginosa* has developed the ability to infect a wide range of different hosts. In humans, severe *P. aeruginosa* infections are frequently

nosocomial and associated with compromised host defences such as neutropenia, severe burns and CF (Lyczak et al., 2000). Of particular interest is the lung infection of individuals suffering from CF. Among all pathogens infecting lungs of CF patients, *P. aeruginosa* strains are considered the most common (isolated from 80% of CF patients – Figure 1,1) and of these people, 25% are infected by multi-drug resistant strains (Foundation, 2012).

P. aeruginosa infections related with airways can be acute or chronic. Acute lung infections usually occur in patients with ventilator-associated pneumonia (VAP). These patients have epithelium damage induced by the insertion of the endotracheal tube, which can itself serve as a reservoir of *P. aeruginosa* (Williams et al., 2010).

The high incidence of *P. aeruginosa* in healthcare institutions is mainly contributed by the presence of multidrug resistant (MDR) strains and prior use of broad spectrum antibiotics (Otter et al., 2011). As a consequence, the VAP infections result in a high mortality rate for vulnerable patients as a result of old age, neutropenia due to cancer chemotherapy or immunosuppression due to treatment for organ transplantation (Williams et al., 2010). Once the infection is well established and if not eradicated properly, *P. aeruginosa* can adapt to the host environment and grow as a biofilm generating chronic infections.

The most relevant chronic infections are those associated with lungs of CF patients, where a thickened Airway Surface Liquid (ASL) is produced due to a mutation in the Cystic Fibrosis Transmembrane Regulator (CFTR). *P. aeruginosa* then colonises the altered ASL causing an initial acute infection which develops into chronic infection following bacterial adaptation and the lowering of the immune response due to the altered ASL (Williams et al., 2010, Sadikot et al., 2005). This can lead to the destruction of lung function, causing progressive deterioration and making these infections lethal.

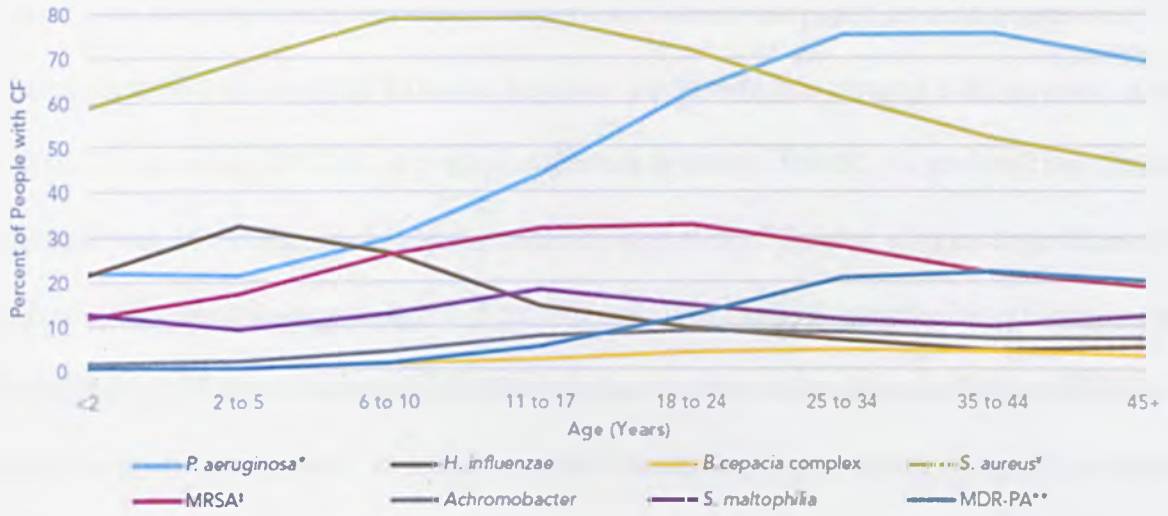


Figure 1.1 Micro-organisms isolated from lungs of Cystic Fibrosis patients in 2012. Graph representing the main micro-organisms isolated from lungs of CF patients in 2012. Among all species isolated, *S. aureus* and *P. aeruginosa* are the most common in lungs of people with CF. The first is present in 80% of the cases in patients aged between 6 and 17, whereas *P. aeruginosa* is present in 80% of the CF patients aged between 24 and 45+. Within this older group, almost 25% are infected by multi-drug resistant *P. aeruginosa* strains (MDR-PA). Figure taken from 2012 Annual Data Report of Cystic Fibrosis Foundation Patient Registry.

Several studies have monitored *P. aeruginosa* infection in CF patients over many years showing that genetic and phenotypic changes occur in this pathogen during chronic infections (Smith et al., 2006, Hogardt et al., 2007, Tingpej et al., 2007, Mena et al., 2008). These changes include loss of flagella and pili (necessary for the adherence, motility and injection of type 3 secreted toxins), mutation of *mucA*, *mucB* and *mucD* genes (involved in the formation of mucoid colonies that may protect the bacterial cells from the innate immune response), development of antibiotic resistance (mostly due to prolonged antibiotic exposure) and alteration of Quorum Sensing (QS) (Winstanley and Fothergill, 2009). For these reasons, strains from chronic infections are less virulent than strains from acute infections when used to infect mice (Bragonzi et al., 2009).

Although *P. aeruginosa* strains isolated from chronic infections have been shown to be less inflammatory and less cytotoxic, they are still extremely serious because they are difficult to eradicate. This can be also increased by a higher level of antibiotic resistance, an environmental adaptation by this bacterium following antibiotic treatments.

1.3. *P. aeruginosa* resistance to antibiotics

Antimicrobial drug resistance represents the acquisition of the capacity of a microorganism to resist the effect of a chemotherapeutic agent to which it is normally susceptible. Whilst The introduction of antibiotics for treating bacterial infections has been successful in overcoming infection, it has also resulted in the development of highly sophisticated resistance strategies in some microorganisms. This has led to the appearance of so called "superbugs" which are resistant to practically all antibiotics available today.

P. aeruginosa has become a superbug and, compared with other pathogens, it is extremely difficult to treat and eradicate because of its already high intrinsic resistance to many antibiotics such as aminoglycosides, β-lactams and fluoroquinolones (Lister et al., 2009, Mesaros et al., 2007). This is mainly due to the presence of an outer membrane

which has low permeability, which reduces the antibiotic penetration into the bacterial cells (Nicas and Hancock, 1983).

In addition to the intrinsic resistance system, which is part of its genetic background, *P. aeruginosa* is able to acquire new resistance traits, which can stabilize and enhance the previous mechanisms (Fernández et al., 2011). This is the case of horizontal transfer of resistant determinants and the occurrence of mutational events, which contribute to develop a very high baseline minimal inhibitory concentrations (MIC) for *P. aeruginosa*.

In addition to the more traditional intrinsic and acquired resistance mechanisms, *P. aeruginosa* can develop adaptive resistance to antimicrobials because of its ability to adapt to changing environments and stress conditions, such as exposure to antibiotics, altered media and growth conditions (Fernández et al., 2011).

1.4. Intrinsic resistance

Compared with other bacteria, *P. aeruginosa* exhibits a higher antibiotic resistance, which makes this pathogen resistant to most of the antimicrobial agents available on the market. This intrinsic tolerance is mainly associated with the nature of its outer membrane, which allows *P. aeruginosa* to survive to a wide range of antimicrobials (McDonnell and Russell, 1999).

The outer layer of the Gram-negative membrane is composed primarily of lipopolysaccharide (Figure 1.2), which forms a hydrophilic barrier against the effects of toxic agents (Nikaido and Vaara, 1985). Additionally, the outer membrane of *P. aeruginosa* is between 12- and 100-fold less permeable compared to other Gram negative bacteria because of its large exclusion limit due to the limited number of large channels of its major porin OprF and the small size of the channels of the other porins, OprD and OprB (Hancock, 1998, Hancock and Nikaido, 1978, Nicas and Hancock, 1983).

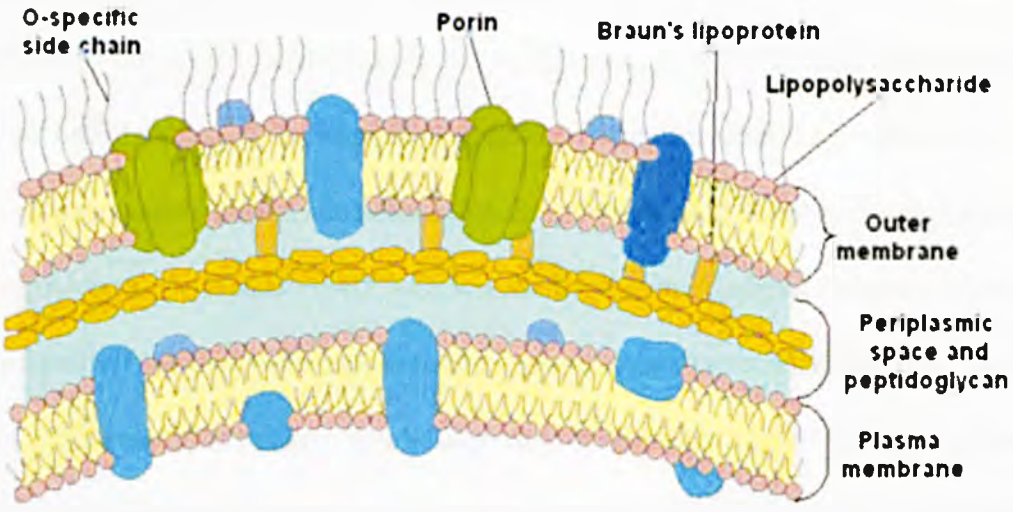


Figure 1.2 Cellular envelope of Gram-negative bacteria.
Figure showing the structure of cell membrane in Gram-negative bacteria. The cellular envelope is characterized by a cytoplasmatic membrane, a periplasmic space and an outer membrane. The outer membrane is mainly composed by lipopolysaccharides, which make the outer layer highly hydrophobic and, as a consequence, reduce the penetration of exogenous hydrophilic molecules. Figures taken from Microbiology Spring 2010 (https://microbiologyspring2010.wikispaces.com/).

Although the penetration of antibiotics is limited, equilibration of hydrophilic molecules across the outer membrane is still manageable. This indicates that the high resistance of this pathogen depends also on other intrinsic and adaptive secondary mechanisms such as rapid efflux, due to intrinsic or induced expression of MexAB-OprM and MexXY-OprM systems (groups of membrane fusion proteins responsible for the efflux of cytotoxic molecules from the cells) , as well as production of AmpC β-lactamases (Li et al., 1995, Li et al., 2000, Masuda et al., 1999).

In addition to the well-known resistance mechanisms, screening of comprehensive mutant libraries has identified new genetic traits, which, when mutated, lead to a decrease in antibiotic resistance, suggesting that more genes may be involved in the intrinsic resistome of *P. aeruginosa* (Fajardo et al., 2008, Alvarez-Ortega et al., 2010, Breidenstein et al., 2008, Dötsch et al., 2009, Schurek et al., 2008, Brazas et al., 2007). These data show that genes from different functional classes can be involved in the development of antibiotic resistance. For example, strains that have mutations in genes affecting DNA replication, recombination and repair shown increased susceptibility to ciprofloxacin (Dötsch et al., 2009, Breidenstein et al., 2008). Similarly, mutations for the gene *ftsK*, involved in cell division processes, and genes involved in alginate production have been shown to be involved in ciprofloxacin and β-lactam resistance (Dötsch et al., 2009, Alvarez-Ortega et al., 2010).

1.4.1. Acquired resistance

The level of *P. aeruginosa* intrinsic resistance to antibiotics, can become even higher following the acquisition of inheritable traits via horizontal transfer of genetic elements or mutations. Events like conjugation, transformation and transduction can lead to the integration of genetic elements (plasmids, transposons, integrons and resistance islands) that can generate single or sometimes multiple antibiotic resistance. This has been

shown for aminoglycoside modifying enzymes, which are located on mobile genetic elements and which are able to chemically modify these molecules and consequently reduce the affinity between aminoglycosides and the 30S ribosomal subunit, which is the target of this class of antibiotics (Vakulenko and Mobashery, 2003). A study has also shown that some *P. aeruginosa* strains have acquired plasmids encoding for new β-lactamases that confer resistance to penicillins and cephalosporins (Sacha et al., 2008).

The second form of acquired resistance, named mutational resistance, is represented by spontaneous mutations which lead to increased resistance to various antimicrobial agents. Mutations can occur with different frequency and can increase in the presence of DNA-damaging agents or growth in a biofilm, where DNA damage is more frequent. For example, the mutation frequency leading to resistance to the antibiotic meropenem has been observed to increase 10-fold in the presence of sub-inhibitory concentrations of ciprofloxacin (Tanimoto et al., 2008).

Strains mutated in genes involved in the repair of DNA replication errors and strains growing in biofilms, show an increase of mutation frequency between 70 and 100 fold (Driffield et al., 2008, Wiegand et al., 2008). The two genes *mutL* and *mutS*, are commonly found to be mutated in *P. aeruginosa* infections in CF patients. These strains can acquire resistance to several antibiotics through mutations to these genes and could play a role during the initial stages of lung infections (Oliver et al., 2000, Kenna et al., 2007).

Another group of mutations that makes *P. aeruginosa* more resistant to antibiotic treatments are those leading to the overexpression of efflux pumps, hyperproduction of β-lactamases and alteration of antibiotic targets. It has been shown that all of these genetic alterations lead to single or multiple resistance to several important antibiotics such as aminoglycosides, fluoroquinolones, β-lactamas, cefepime and imipenem (Stickland et al., 2010, Muller et al., 2011, Walsh and Amyes, 2007, Sobel et al., 2005, Hooper, 2001,

Dunham et al., 2010).

The data available on acquired resistance mediated by mutations indicates that such mutations occur in a large number of unrelated genes and can give rise to acquired resistance to different antibiotics. For example, the aminoglycoside resistome involves 150 different genes related to energy metabolism, DNA replication and repair and lipopolysaccharide (LPS) biosynthesis (Dötsch et al., 2009, Schurek et al., 2008). The fluoroquinolone resistome involves NADH dehydrogenase genes, phage-related genes, iron transport genes and efflux regulators (Dötsch et al., 2009, Breidenstein et al., 2008). Resistance to β-lactams involves genes that can affect efflux systems, overproduction of β-lactamase and LPS biosynthesis (Dötsch et al., 2009, Alvarez-Ortega et al., 2010).

All screening studies for mutations that affect *P. aeruginosa* resistance have demonstrated that the resistomes for the main antibiotics available involve a wide variety of mutations of a large number of genes and often from different functional categories. These findings highlight the difficulties in finding reliable new targets for antimicrobial treatments for *P. aeruginosa* infections.

1.4.2. Adaptive resistance

Adaptive resistance is a form of resistance induced in the presence of particular environmental stimuli. Although this kind of resistance has been known for a long time, the molecular basis has not been established yet (Barber and Waterworth, 1966). However, with new genomic approaches, it has been shown that factors triggering adaptive resistance include antibiotics, polyamines, pH, anaerobiosis as well as changes in carbon sources for growth (Fernández et al., 2011). This is more evident considering the large number of regulatory genes present in the *P. aeruginosa* genome (8.4%), which modulate their expression in specific environmental conditions (Stover et al., 2000). This indicates that *P. aeruginosa* cells exposed to antibiotics, generally at sub-inhibitory concentrations, are able

to change their gene expression pattern allowing the cells to survive when subsequently exposed to lethal concentrations of the same or related antibiotics. Interestingly, when the resistance inducing factor is removed, the cells revert to wild type, explaining why results in *vitro* are often not usable to predict those in *vivo* (Fick and Stillwell, 1989). Some studies have shown that exposure of *P. aeruginosa* to commonly used antibiotics such as colistin, tobramycin, β-lactams and aminoglycosides leads to the overexpression of genes (*arn*, AmgRS two component regulator, *ampC*, Lon protease and MexXY efflux pump) which in turn results in increased antibiotic resistance (Fernández et al., 2010, Sanders, 1993, Lee et al., 2009, Marr et al., 2007, Hocquet et al., 2003).

Increase in antibiotic resistance has been also observed in cells of *P. aeruginosa* growing in biofilms. Comparison of transcriptomic data between planktonic cells and cells growing in a biofilm have shown significantly different patterns of gene expression across a wide range of genes (Whiteley et al., 2001). As a result, *P. aeruginosa* cells show remarkable phenotypic changes such as increasing antibiotic resistance (Costerton et al., 1999, O'Toole and Kolter, 1998). Although the effect of biofilm growth on antibiotic resistance is well documented, the identification of specific gene targets responsible for this phenomenon is still difficult because the broad dysregulation of genes belonging to many different functional categories.

Swarming motility is another adaptation feature in *P. aeruginosa*. It is a form of motility that plays an important role in colonization, attachment to surfaces and biofilm formation and, as in biofilm growth, involves dysregulation of several genes (Overhage et al., 2008, O'Toole and Kolter, 1998). Swarming is thought to be relevant in CF as it is necessary for cell mobility through mucosal layers, an environmental condition present in lungs of CF patients (Hutchison and Govan, 1999). Although *P. aeruginosa* swarming colonies show higher expression of virulence factors and higher antibiotic resistance, the correlation between adaptive resistance and swarming motility has not been clarified yet

(Overhage et al., 2008, Lai et al., 2009).

All the data related to intrinsic, acquired and adaptive resistance (Table 1.1) show that *P. aeruginosa* resistance is the result of a combination of multiple mechanisms acting at the same time and which make this pathogen extremely resistant to many antimicrobial agents.

Table 1.1 Overview of the *P. aeruginosa* resistance.
Overview of the *P. aeruginosa* resistance showing the mechanisms and example of genes involved in the intrinsic, acquired and adaptive resistance of this pathogen.

Type of resistance	Mechanism/process	Example of genes involved
Intrinsic	Outer membrane	*mexA, mexB, OprF* (Dötsch et al., 2009)
	β-lactamases production	*ampC, ampG* (Masuda et al., 1999, Zhang et al., 2010)
	Over expression of efflux pumps	*mexR, mexZ,* (Masuda et al., 1999, Li et al., 1995, Li et al., 2000)
	Cell division	*ftsK* (Dötsch et al., 2009)
	DNA recombination, replication and repair	*sss, recG* (Breidenstein et al., 2008)
Acquired	Horizontal transfer of genetic elements	*bla,* APCs, ACCs, ANTs (Sacha et al., 2008, Vakulenko and Mobashery, 2003)
	Mutations	*mutL, mutS, mexR, nfxB* (Oliver et al., 2000, Stickland et al., 2010, Muller et al., 2011)
Adaptive	Gene expression change	*arn* (Fernández et al., 2010)
	Over expression of efflux pumps	*mexZ* (Hocquet et al., 2003)
	Protein expression	*lon* (Marr et al., 2007)

1.5. Correlation between virulence and antibiotic resistance in P. aeruginosa

Virulence and antibiotic resistance are often correlated in bacteria, and it is known that some regulatory genes are involved in both processes. In *P. aeruginosa* some genes involved in virulence are often controlled by environmental factors typically associated with the host environment (Yeung et al., 2011, Linares et al., 2010, Hoffman et al., 2005, Gooderham et al., 2009a). Virulence genes are involved in virulence factor production, cytotoxicity, swarming motility as well as being identified in biofilm formation (Bjarnsholt et al., 2009). Considering that in *P. aeruginosa* some virulence genes are involved during swarming motility and biofilm production and that both bacterial behaviours lead to increased antibiotic resistance, virulence and resistance can be in some cases be considered to be correlated in this bacterium (Overhage et al., 2008, Linares et al., 2010, Gooderham et al., 2009a, Høiby et al., 2010, Macfarlane et al., 2000).

Some examples of the relationship between virulence and resistance in *P. aeruginosa* are described below. The gene *Lon*, which was observed to be involved in fluoroquinolone resistance, regulates the RhlR-RhlI and LasR-LasI QS systems which control virulence (Breidenstein et al., 2008, Brazas et al., 2007, Takaya et al., 2008). The same gene is expressed during swarming and in the presence of aminoglycosides, antibiotics that also induce biofilm formation (Marr et al., 2007, Hoffman et al., 2005). These data indicate that this gene is involved in adaptive resistance as well as virulence.

The gene *P. aeruginosa psrA* is overexpressed in the presence of antimicrobial peptides and fatty acids, molecules which lead to an increased antibiotic resistance (Gooderham et al., 2008, Kang et al., 2008). It has been shown that *psrA* also effects the expression of genes involved in swarming and biofilm production (Kojic et al., 2002, Kang et al., 2009), whereby a *psrA* mutant has shown biofilm and swarming deficient

phenotypes (Gooderham et al., 2008).

Mutants for the sensor kinase *CbrA*, a gene involved in nitrogen- and carbon-metabolism, show increased resistance to ciprofloxacin, colistin and tobramycin as well as reduced swarming motility and increased biofilm production in *P. aeruginosa* (Yeung et al., 2011).

Another example of relationship between virulence and antibiotic resistance is represented by the *P. aeruginosa* gene *PhoQ*. When this gene is mutated, constitutive expression of the *arn* operon (involved in the reduction of the self-promoted up-take of molecules into the cells) is observed leading to resistance to various antimicrobials (Gooderham et al., 2009a, Macfarlane et al., 2000). Compared with *P. aeruginosa* wild type, *PhoQ* mutants also show a reduction in motility and biofilm formation, less pyocyanin production (blue redox-active secondary metabolite produced by *P. aeruginosa* during infections) and less cytotoxicity and resulting less virulent on mouse model of chronic lung infection (Gooderham et al., 2009a).

All of these data clearly prove that some genes can be involved in both virulence and antibiotic resistance processes in *P. aeruginosa*. In particular, swarming motility and biofilm production, which have been extensively characterized as virulence related processes, show to be also correlated with antibiotic resistance. This highlights the need for monitoring these two bacterial behaviours during antibiotic resistance studies.

1.6. Azoreductases

Azoreductases, a group of flavoenzymes that catalyse the reductive cleavage of azo bonds (-N=N-) in azo compounds, are found ubiquitously in different bacterial species such as *E. coli*, *Entrerococcus faecalis*, *Sinorhizobium meliloti*, *Bacillus subtilis*, *Rhodobacter sphaeroides*, *P. aeruginosa* as well as in yeast and humans (Nakanishi et al., 2001, Chen et al., 2004, Ye et al., 2007, Sugiura et al., 2006, Liu et al., 2007, Wang et al., 2007, Sollner et al., 2007, Cui et al., 1995). Some bacterial species that live in the human gastrointestinal tract have been described as expressing azoreductases (Chung et al., 1978, Brown, 1981, Nakamura et al., 2002).

Azo compounds to which humans are generally exposed to are azo dyes and azo pro-drugs, which are used as Inflammatory Bowel Disease (IBD) treatments (Bäumler et al., 2000, Collins and McLaughlin, 1972, Dissanayake and Truelove, 1973). Azo pro-drugs such as sulfasalazine and balsalazide have been developed by cross-linking either an inert carrier molecule or a molecule of 5-aminosalicylate itself via an azo bond to a molecule of 5-aminosalicylic acid (5-ASA) which is reductively cleaved in situ by azoreductase enzymes (Klotz, 2005, Svartz, 1988, Brown et al., 1983, Chan et al., 1983, Willoughby et al., 1982). With this mechanism, azo pro-drugs are activated when azoreductases expressed by human intestinal flora cleave the azo bond between carrier and 5-ASA (Peppercorn and Goldman, 1972). Azoreductases have also been studied for their ability to reduce azo dyes and their possible use in the bioremediation of these compounds generated during the production processes of range of industries including textiles and printing (Mahmood et al., 2015, Mendes et al., 2011a, Lang et al., 2013).

Homology studies with an azoreductase from *E. coli* (AzoR) have led to the identification and characterization of three azoreductase (paAzoR1-3) in *P. aeruginosa* (Wang et al., 2007, Ryan et al., 2010a). Wang and colleagues and subsequently Ryan and

colleagues have shown the presence of flavin mononucleotide (FMN) molecules non-covalently bond to the paAzoR1-3 proteins via absorbance spectra analysis and protein crystal structure determination (Figure 1.3) (Wang et al., 2007, Ryan et al., 2010b). Additionally it has been shown that all three enzymes use NAD(P)H as the electron donor for the reduction of azo compounds, consistent with data obtained for azoreductases from other bacteria (Ryan et al., 2010b, Wang et al., 2007, Nakanishi et al., 2001, Deller et al., 2006).

The reaction mechanism of these enzymes has been proposed showing that the reduction of azo substrates follows a characteristic ping pong bi bi mechanism (Nakanishi et al., 2001, Wang et al., 2010). The proposed reduction mechanism of balsalazide by *P. aeruginosa* PAO1 paAzoR1 shows that this mechanism involves two cycles of reduction of flavin, which reduces the azo substrate to a hydrazine in the first cycle, and the hydrazine to two amines in the second cycle (Figure 1.4). In particular, the FMN molecules, which are non-covalently bond to the glycine 146 and 147 of the enzyme, are reduced from FMN to $FMNH^-$ by NADPH. The reduced flavin ($FMNH^-$) then transfers two electrons to the substrate, which forms the hydrazine intermediate. Under acid conditions, the azo bond -N=N- of the intermediate is cleaved, with the formation of an amine and an iminoquinone. The iminoquinone undergoes to a second reduction cycle with the formation of the second amine (Ryan et al., 2010a).

Because the reduction of one molecule of azo substrate requires four electrons and one molecule of NAD(P)H + H is a two-electron carrier, two molecules of NAD(P)H are required to complete the reaction indicating that the azo substrates reduction is likely to correspond to a double ping pong bi bi reaction mechanism (Wang et al., 2007, Nakanishi et al., 2001, Ito et al., 2006).

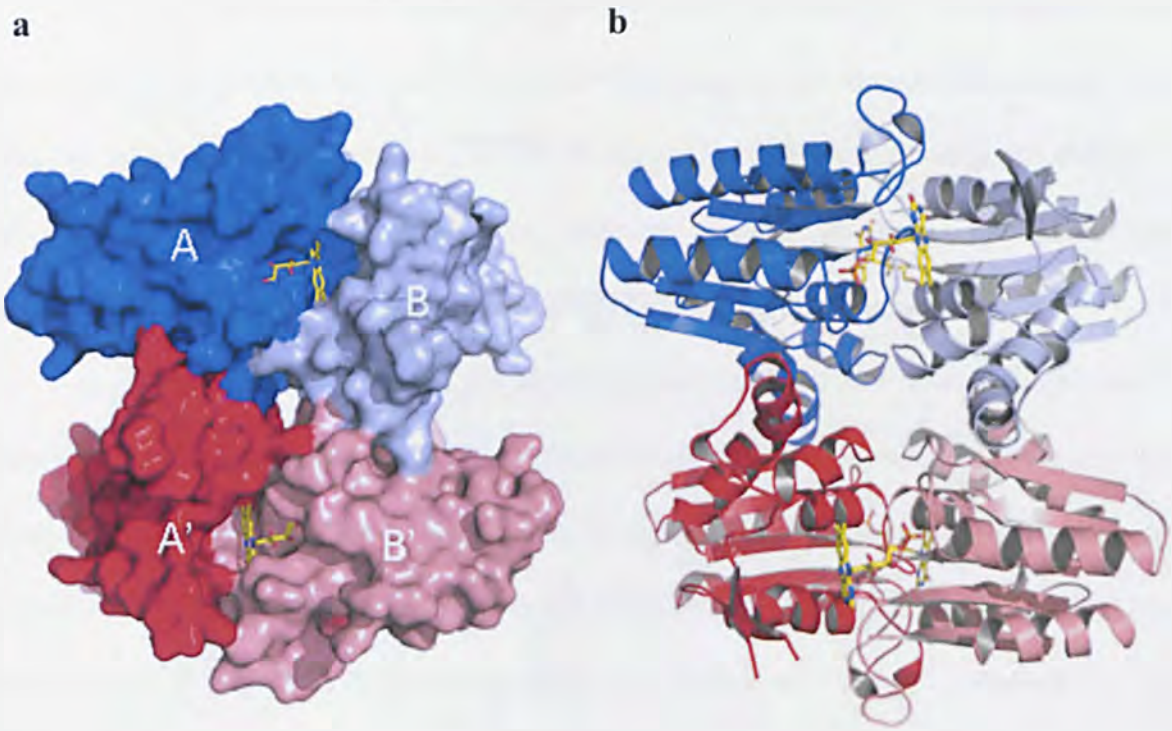


Figure 1.3 paAzoR1 crystal structure with FMN
(**a**) Molecular surface representation of paAzoR1 tetramer which can be subdivided into two functional dimers (blue and in red) containing FMN molecules (yellow). (**b**) Ribbon diagram of the tetramer containing four molecules of non-covalently bound FMN. In the tetramer, each dimer is related to its partner dimer by a 2-fold crystallographic axis, and the monomers in the dimers are related to each other by a non-crystallographic axis. Figure taken from Wang et al. (2007). Protein Data Bank (PDB) ID: 2V9C.

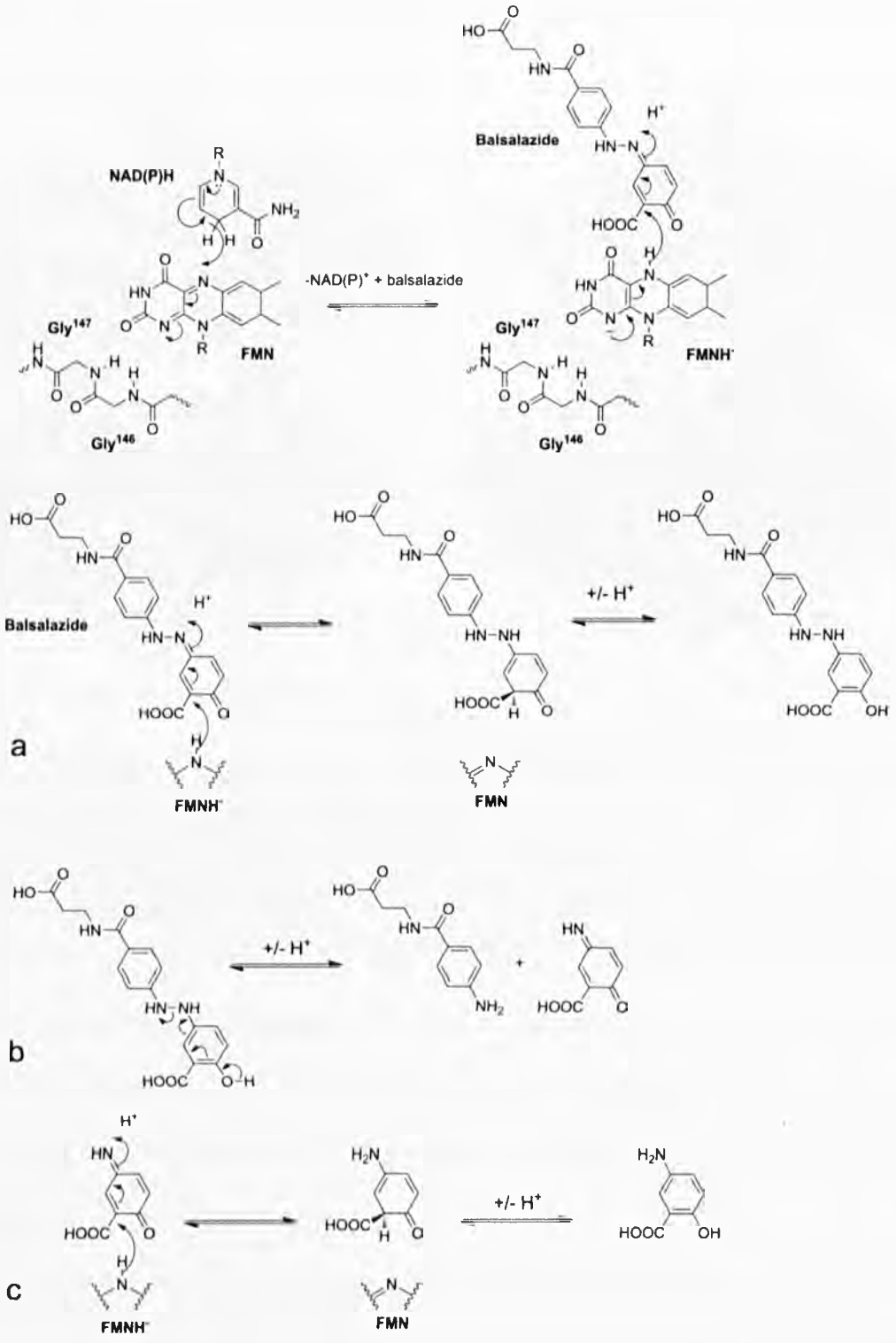


Figure 1.4 Proposed reduction mechanisms of the azo compound balsalazide.
Figure showing the (**a**) reduction of FMN to $FMNH^-$ by NADPH and subsequent hydrade transfer to balsalazide, (**b**) theoretical mechanism for the breakdown of the hydrazine intermediate and (**c**) theoretical mechanism for the reduction of iminoquinone. Figure taken from Ryan et al. (2010).

The enzymatic characterization of the three azoreductases from *P. aeruginosa* has shown that these enzymes are able to reduce a wide range of azo compounds although with different reduction rates (Ryan et al., 2010b, Wang et al., 2007). Additionally, the ability of azoreductases to reduce a variety of different compounds has complicated the identification of their physiological substrate and hence their function.

A recent study showed that the same enzymes are also able to reduce quinones, although it was thought that NAD(P)H quinone oxidoreductases (NQOs) were a distinct group of enzymes, which have been found in many different species from bacteria to mammals, similarly to the azoreductase enzyme (Ryan et al., 2014, Nakanishi et al., 2001, Mendes et al., 2011b, Chen et al., 2005a, Wu et al., 1997).

As previously described for azo compounds, the reduction of quinones also involves electron transfer from NAD(P)H molecules, although only two electrons are necessary to reduce one molecule of quinone whilst four are required for reduction of azo compounds (Chesis et al., 1984). Also in the case of quinones, paAzoR1-3 from *P. aeruginosa* was shown to reduce a wide range of these compounds such as naphtoquinones, benzoquinones and quinonemines (Ryan et al., 2014). Additionally, the three identified azoreductase enzymes from *P. aeruginosa* show higher rate of reduction with quinones than with azo compounds, suggesting that their primary substrate preference is for quinones. This may be explained by the fact that azo compounds are not commonly found in nature, whereas quinones are produced by many organisms.

One of the reasons why azoreductases and NQOs have been considered as two distinct group of enzymes is because of the protein sequence diversity among the members. The original three characterised azoreductases paAzoR1 paAzoR2 and paAzoR3 (PA0785, PA1962 and PA3223 respectively), were identified via sequence homology with the *E. coli* AzoR enzyme using a 30% homology cut-off, because of the low sequence identity (Wang et al., 2007). This, together with experimental data, suggests that proteins with very low

sequence homology can have very similar enzymatic activities. However, the identification of new members of this group is still difficult using traditional sequence homology analysis.

A combination of bioinformatics approaches, including comparison of structural, enzymatic and sequence homology data from many different species, were applied in the identification of seven new putative azo- and quinone- reductase enzymes in *P. aeruginosa* PAO1 (Table 1.2) (Ryan et al., 2014).

Although the enzymatic activities of azoreductases from *P. aeruginosa* PAO1 and other bacterial species have been extensively described, their physiological role still remains unclear. Considering the higher quinone oxidoreductase activities of these enzymes, it has been proposed that they may participate in *P. aeruginosa* infection processes such as the detoxification of cytotoxic quinones produced as antibacterial compounds by many plants (Ryan et al., 2014). This hypothesis is supported by studies showing evidence of azoreductases been involved in infection processes in both plants and animals (Goudeau et al., 2013, Landstorfer et al., 2014, Rakhimova et al., 2008, Skurnik et al., 2013). Interestingly, it has also been shown that a putative azoreductase gene (*pa2580*) is involved in *P. aeruginosa* antibiotic resistance (Chen et al., 2010b). Although these data do not completely clarify the physiological role of azo- and quinone- reductase enzymes, they strongly suggest that these enzymes may be involved in important processes such as bacterial pathogenesis and resistance in *P. aeruginosa*.

Although the enzymatic activities of azoreductases from *P. aeruginosa* PAO1 and other bacterial species have been extensively described, their physiological role still remains unclear.

Table 1.2 New putative *P. aeruginosa* PAO1 azoreductase proteins identified.
Details of putative *P. aeruginosa* PAO1 azoreductase proteins identified based on sequence homology, enzymatic function and structural data of azoreductases from other organisms by Ryan and colleagues (Ryan et al., 2014). Annotated functions were taken from Pseudomonas database (Winsor et al., 2011). Homologues showing azo- and/or quinone oxidoreductase function include ecWrbA (*E. coli*), atNQO (*A. thaliana*) hNQO1 (human), hNQO2 (human), smArsH (*S. meliloti*), ecMdaB (*E. coli*) and hpMdaB (*H. pylori*).

Protein ID	Annotated function	Homologues	Homologues azo/quinone reduction
PA0949	Trp repressor binding protein	ecWrbA	Quinone oxidoreductase (Patridge and Ferry, 2006)
PA1204	NAD(P)H quinone oxidoreductase	atNQO	Quinone oxidoreductase (Sparla et al., 1999)
PA1224	Probable NAD(P)H dehydrogenase	hNQO1 and hNQO2	Azo- and quinone oxidoreductase (Wu et al., 1997)
PA1225	Probable NAD(P)H dehydrogenase	hNQO1 and hNQO2	Azo- and quinone oxidoreductase (Wu et al., 1997)
PA2280	Oxidoreductase	smArsH	Azoreductase (Hervas et al., 2012)
PA2580	Conserved hypothetical protein	ecMdaB and hpMdaB	Quinone oxidoreductase (Hayashi et al., 1996, Wang and Maier, 2004)
PA4975	NAD(P)H quinone oxidoreductase	hNQO2	Azo- and quinone oxidoreductase (Wu et al., 1997)

Considering the higher quinone oxidoreductase activities of these enzymes, it has been proposed that they may participate in *P. aeruginosa* infection processes such as the detoxification of cytotoxic quinones produced as antibacterial compounds by many plants. This hypothesis is supported by studies showing evidence of azoreductases been involved in infection processes in both plants and animals. Interestingly, it has also been shown that a putative azoreductase gene (*pa2580*) is involved in *P. aeruginosa* antibiotic resistance. Although these data do not completely clarify the physiological role of azo- and quinone-reductase enzymes, they strongly suggest that these enzymes may be involved in important processes such as bacterial pathogenesis and resistance in *P. aeruginosa*.

1.7. Aims

Today the presence and the number of *P. aeruginosa* MDR strains constitute one of the major health concerns for people suffering from CF. *P. aeruginosa* is a highly adaptable pathogen with an intrinsic ability to resist a large number of antimicrobial agents which makes successful treatment very difficult. Furthermore, it has been demonstrated that within the infected host, bacteria reside in biofilms, a condition which makes *P. aeruginosa* even more resistant to antibiotic treatment. For these reasons, identifying new targets for therapy has become extremely important.

A number of studies have shown evidences on the possible role of *P. aeruginosa* azoreductases on host infection and antibiotic resistance processes in this pathogen. Therefore, identifying new azoreductases and understanding their molecular and physiological role(s) is a crucial step toward for the development of new antibiotic treatments to use for *P. aeruginosa* infections and in particular for CF patients.

In particular, the aims of this study are:

1. Sequencing and cloning of putative *P. aeruginosa* PAO1 azoreductase genes
2. Production and purification of putative *P. aeruginosa* PAO1 azoreductases as recombinant proteins
3. Biochemical and enzymatic analyses of putative *P. aeruginosa* PAO1 azoreductase recombinant proteins
4. Analysis of *P. aeruginosa* PAO1 biofilm production and swarming motility using azoreductase single gene transposon mutants
5. Analysis of *P. aeruginosa* PAO1 antibiotic resistance using azoreductase single gene transposon mutants

Chapter 2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in this study were supplied by Sigma-Aldrich unless specified. DNA primers were synthesised and supplied also by Sigma-Aldrich. All DNA extraction and purification kits and Taq polymerase enzymes were supplied by Qiagen unless specified. Restriction enzymes were supplied by New England Biolabs (NEB).

2.2. Bacterial strains and growth conditions

In this study *P. aeruginosa* PAO1 wild type and a set of single gene transposon mutants were used. All mutant strains were generated and provided by Manoil laboratory, University of Washington (Jacobs et al., 2003). *E. coli* JM109ES and BL21 strains used for cloning and recombinant protein production were provided by Promega. Details of all bacterial strains used are shown in table 2.1.

The Growth media were prepared following the supplier instructions and sterilized following the standard media sterilization procedure (121°C for 20 minutes), unless specified. Antibiotics were prepared following the suppliers instructions and stored at -20° C. These were always added, when needed, after the sterilization process. Media details are provided in table 2.2. *P. aeruginosa* PAO1 wild type *E. coli* strains (containing no plasmid) were grown in of Luria Bertani (LB) broth or LB agar overnight at 37° C (180 rpm shaking with liquid medium) as described by Wang and colleagues (Wang et al., 2007).

P. aeruginosa PAO1 transposon mutants and *E. coli* strains containing pET28b+ plasmid (Novagen), were grown as described above and adding 12.5 μg/ml of tetracycline or 30 μg/ml of kanamycin respectively.

Table 2.1 Details of *P. aeruginosa* PAO1 and *E. coli* stains used.
Table showing the bacterial strains used in this study and their corresponding gene mutated. *P. aeruginosa* PAO1 wild type and single gene transposon mutants were provided by University of Washington (UW). *E. coli* strains used for cloning (JM109ES) and recombinant protein production (BL21) were provided by Promega.

Strain	Gene mutated	Supplier
P. aeruginosa PAO1	-	UW
P. aeruginosa PAO1	*paazor1*	UW
P. aeruginosa PAO1	*paazor2*	UW
P. aeruginosa PAO1	*paazor3*	UW
P. aeruginosa PAO1	*pa0949*	UW
P. aeruginosa PAO1	*pa1204*	UW
P. aeruginosa PAO1	*pa2280*	UW
P. aeruginosa PAO1	*pa2580*	UW
P. aeruginosa PAO1	*pa4975*	UW
E. coli JM109ES	-	Promega
E. coli BL21	-	Promega

Table 2.2 Media used in this study.
Table showing all media used in this studying, their components and concentrations.

Medium	Components	Concentration (g/l)
LB broth	tryptone NaCl yeast extract	10 10 5
LB agar	LB broth Bacto agar	 15
M9 minimal medium	Na_2HPO_4 KH_2PO_4 NaCl NH_4Cl D(+)-glucose $MgSO_4$ $7H_2O$ $CaCl_2$ $2H_2O$ Thiamine $FeSO_4$ $7H_2O$	12.8 3 0.5 1 2 0.494 0.0152 0.01 0.01
M9 minimal medium agar	M9 minimal medium Bacto agar	 15
BHI broth	beef heart calf brain Na_2HPO_4 D(+)-glucose peptone	5 12.5 2.5 2 10
BHI agar	BHI broth Bacto agar	 15
Mueller Hinton broth	beef infusion solids casein hydrolysate starch	2 17.5 1.5

2.2.1. Preparation of glycerol stocks

For long term storage, all bacterial strains were stored as glycerol stocks at -80° C. *P. aeruginosa* PAO1 wild type and single gene transposon mutants were received in stab cultures in LB agar medium. These were used to inoculate fresh LB agar plates and and growth as described in section 2.2. Single colonies were selected and grown in LB broth as described in section 2.2. After the incubation period (approximately 16 hours), cells were collected by centrifugation at 4500g for 1 minute and resuspended with fresh LB broth containing 15% (v/v) sterile glycerol. Samples were then transferred in 2 ml cryovials, flash frozen in liquid nitrogen and stored at -80° C. The same procedures were also used to generate glycerol stocks of the *E. coli* (JM109 and BL21) strains transformed with plasmids containing azoreductase and putative azoreductase genes.

2.3. Sequencing of putative *P. aeruginosa* PAO1 azoreductase genes

2.3.1. Genomic DNA extraction and quantification

P. aeruginosa PAO1 wild type was grown in LB broth as previously described (section 2.2) and an overnight culture was used to extract the total genomic DNA using Gentra Puregene Yeast/Bact. Kit following the supplier instructions. DNA samples were analysed and quantified using NanoVue™ Plus Spectrophotometers (VWR).

2.3.2. Gene amplifications

The genes *pa0949, pa1204, pa2280, pa2580,* and *pa4975* were amplified via PCR using the previously extracted *P. aeruginosa* PAO1 wild type genomic DNA (section 2.3.1). Specific primers were designed approximately 50 bp upstream (Forward) and downstream (Reverse) of the coding region of each gene of interest (Table 2.3), as the sequencing reactions give reliable results only 20-30 nucleotides after the primer sequences.

Table 2.3 Gene specific primers used for the amplification of putative *P. aeruginosa* PAO1 azoreductase genes for the sequencing process.

Table showing the forward (Fwd) and reverse (Rev) primers used to amplify putative *P. aeruginosa* PAO1 azoreductase genes to sequence. Each primer was designed approximately 50 bp upstream (Fwd primer) and 50 bp downstream (Rev primer) of the gene coding region of each gene.

Primer	Sequence (5'-3')	Target gene	Expected PCR product (bp)
pa0949seq Fwd	GAAAGCGATCCTGGACAAGCTCGGCATTCC	*pa0949*	690
pa0949seq Rev	CAGGTTGACCACGTAGTAGGCCCAGGCATG		
pa1204seq Fwd	CTGCATGCCGATCACCGTGGCGATGTACG	*pa1204*	650
pa1204seq Rev	CGACCAGTCCGCCGCCAGGTTCGAATTCG		
pa2280seq Fwd	GCACCACCTGACATCGTCATCACCGTTTGC	*pa2280*	790
pa2280seq Rev	CGGGTTCGGTCGCTCGATGTTGCAGTACG		
pa2580seq Fwd	CTCACATGCGTTCGAATGCTGGCGACCC	*pa2580*	690
pa2580seq Rev	GTGTCTTCGTCACGGTCGAAAGCGCGCTC		
pa4975seq Fwd	CCTGGTAATCGAGTGCATGGCCGACAGC	*pa0949*	800
pa4975seq Rev	GCCGCTACGCCTACATCCTCGATACCCTGC		

All primers were manually designed and analysed for GC content, melting temperature, secondary structures and dimers formation using the Integrated DNA Technologies (IDT) oligo analyzer tool available on line (Owczarzy et al., 2008).

The PCR reaction mix was prepared using the HotStar HiFidelity Taq Polymerase following the supplier instructions. Table 2.4 shows the PCR reaction mix components and the volume used in each reaction. PCR reactions were optimized using different annealing temperatures in order to find the optimum conditions to amplify all genes of interest simultaneously and performed using a MJ Research PTC-200 Peltier Thermal Cycler. The thermocycling conditions used are shown in table 2.5.

2.3.3. PCR products analysis and purification

PCR products were analysed on 1% (w/v) agarose gels in 1X TBE buffer (108 g/l tris base, 55 g/l boric acid, 9.3 g/l EDTA disodium salt, pH 8) with 1% (v/v) SYBR Safe DNA gel stain (Life Technologies). Gel loading dye (30 % v/v glycerol 0.25% w/v bromophenol blue and 69.75% v/v dH_2O) was added to each sample (1:10) and mixed. Ten µl of sample-gel loading dye mix were loaded in each well. A 1 Kb Plus DNA Ladder (Invitrogen) was used as standard. Agarose gels were run for 45 minutes at 90 volts in an electrophoresis chamber (Flowgen) using 1X TBE as running buffer and analysed using Molecular Imager Gel Doc™ XR+gel (BIO-RAD). PCR products were then purified using Minielute PCR Purification kit following the suppliers instruction.

The size of PCR products was determined based on the migration of each band compared with the migration of the standard bands. In particular, the $\log_{10}$ of bp length of the standard bands were calculated and associated to the migration of each band. Linear regression for all bands for the standard was calculated and the $\log_{10}$ of the PCR products lenght was interpolated based on their migration.

Table 2.4 PCR reaction mix used to amplify putative *P. aeruginosa* PAO1 azoreductase genes for the sequencing process.
Table showing the PCR mix components used to amplify putative *P. aeruginosa* PAO1 azoreductase genes from *P. aeruginosa* PAO1 wild type genomic DNA. A PCR product was generated for each target gene and used for sequencing.

Solution	Volume (µl)	Final concentration
Genomic DNA	1	2 ng
Forward primer (10 µM)	5	1 µM
Reverse primer (10 µM)	5	1 µM
HotStar HiFidelity Taq polymerase (2.5U/µl)	2	5U
5X HotStar HiFidelity PCR Buffer (1.5 mM dNTPs, Factor SB and 7.5 mM $MgSO_4$)	10	1X
5X Q-Solution	10	1X
RNase-free water	18	

Table 2.5 Thermocycling condition used to amplify putative *P. aeruginosa* PAO1 azoreductase genes for the sequencing process.
Table showing the thermocycling conditions used to amplify putative *P. aeruginosa* PAO1 azoreductase genes to sequence.

PCR step	Temperature (°C)	Time	N° of cycles
Taq polymerase activation	**95**	5 min.	1
Primers annealing	**55**	30 sec.	35
Fragments extention	**72**	1 min.	
DNA denaturation	**95**	30 sec.	
Primer annealing	**55**	30 sec.	1
Final extention	**72**	10 min.	1

2.3.5. Gene sequencing

The purified PCR products together with the primers used to amplify all genes (section 2.3.2) were sent for sequencing to Eurofins mwg|operon in the volumes and concentrations suggested by the company (Table 2.6). The sequences data obtained with the forward and reverse sequencing reactions were aligned and compared with the *P. aeruginosa* PAO1 *pa0949, pa1204, pa2280, pa2580*, and *pa4975* gene sequences available on Pseudomonas database (Winsor et al., 2011). The multiple sequence alignments were performed using the ClustalW algorithm on Bioedit software (Hall, 1999).

2.4. Cloning of putative *P. aeruginosa* PAO1 azoreductase genes

The putative azoreductase genes *pa0949, pa1204*, *pa2280*, *pa2580*, and *pa4975* were sequenced to verify the correspondence of these with the sequences annotated in the Pseudomonas database as described in section 2.3. These genes were then amplified from *P. aeruginosa* PAO1 wild type and cloned into *E. coli* strains.

2.4.1. Gene amplifications

P. aeruginosa PAO1 wild type genomic DNA was extracted and quantified as described in section 2.3.1. Gene specific primers were designed to overlap part of the coding and part of the non-coding regions, in order to obtain high binding affinity primers. These were designed and analysed as described in section 2.3.2, and including NdeI and SacI restriction sites in the forward and reverse primer sequences respectively (Table 2.7).

The start codon of the gene *pa0949* (TTG) was changed to ATG in order to use NdeI restriction enzyme as for the other genes cloned. NdeI and SacI restriction sites were chosen based on the restriction sites available in pET28b(+) plasmid. NdeI was also chosen because the sequence recognised by this enzyme (CATATG) includes the start codon (ATG) present in all gene sequences to clone.

Table 2.6 PCR products and primers concentrations and volumes suggested by Eurofins mwg|operon for the sequencing process.
Table showing the PCR products as well as reverse and forward primers volume and concentration suggested by Eurofins mwg|operon to perform the gene sequencing.

Solution	Concentration	Volume
Purified PCR product	50 ng/µl	15 µl
Fwd primer	10 pmol/µl	2 µl
Rev primer	10 pmol/µl	2 µl

Table 2.7 Primers used for the amplification of putative *P. aeruginosa* PAO1 azoreductase genes for the cloning process.
Table showing the forward (Fwd) and reverse (Rev) primers used for the amplification of putative azoreductase genes from *P. aeruginosa* PAO1. NdeI (CATATG) and SacI (GAGCTC) restriction sites were included in the Fwd and Rev primer sequences respectively to allow further manipulation with restriction enzymes.

Primer	Sequence (5'-3')	Target gene	Expected PCR product (bp)
pa0949 Fwd	GAGTCCTGGAGATCCTCATATG AGCAGTCCCTACATCCTG	*pa0949*	597
pa0949 Rev	GTGAGCTCTTGCGGGCCATTTC AACTCCCCAGCTTG		
pa1204 Fwd	CGAGGAGCACCATATGAGCGA CGACATCAAGGTATTG	*pa1204*	558
pa1204 Rev	CATGTTCGAGCTCTCCGGTGAC GAGGCATTCAAC		
pa2280 Fwd	CCGGAGGACATATGTCCGAAC AACTACCCAACCTCG	*pa2280*	693
pa2280 Rev	GGCGGACGGAGCTCAGAGCGA ACGCTGGTCGACCC		
pa2580 Fwd	CCAGCATGTACGGAACCATAT GAAAATCATTCTCCTGC	*pa2580*	591
pa2580 Rev	GGCCCGAGCTCGAACTCAGCC GGCGCG		
pa4975 Fwd	CGGAAACCCATATGAACGTAC TGATCGTCCACGC	*pa4974*	705
pa4975 Rev	CGCCGAGCTCAGCGCGCCAGC GGCTGG		

Using NdeI and SacI restriction sites also allows incorporation of a HisTag coding region, which encodes for hexahistidine residues upstream of the gene sequence to clone.

PCR reactions were prepared and performed as described in table 2.4 and 2.5 in section 2.3.2. The PCR products quantification and analysis was performed as described in section 2.3.1 and 2.3.3 and the amplified putative azoreductase genes were then purified as described in section 2.3.3.

2.4.2. Empty plasmid production and isolation

E. coli JM109ES containing the empty plasmid pET28b+ (no insert) was grown as previously described (section 2.2). Plasmid extraction was performed using Qiaprep Spin Miniprep Kit (Qiagen) following the suppliers instructions. Plasmid samples were quantified as described in section 2.3.1.

2.4.3. Restriction digestion of empty plasmids and inserts

Empty pET28b+ plasmid and PCR products of putative azoreductase genes were digested using NdeI and SacI restriction sites (Table 2.8) cutting at the 5' and 3' respectively of both plasmid and inserts. In this way sticky ends at 5' (NdeI) and 3' (SacI) for both insert and plasmid were generated allowing only one possible direction for the insertion of the genes inside the plasmid sequence. Samples were incubated at 37 ºC for one hour followed by enzyme inactivation at 65 ºC for 20 minutes.

Digested and undigested plasmid samples were analysed on 1% (w/v) agarose gel as described in section 2.3.3. Inserts were purified as described in section 2.3.3, whereas plasmid samples were purified using the QIAquick PCR Purification kit (Qiagen) following the suppliers instructions. Both plasmid and inserts were analysed and quantified as previously described (section 2.3.1).

Table 2.8 Plasmid and inserts restriction digestion details.
Tables showing the reaction mix used for the PCR products and plasmid restriction digestion with NdeI and SacI enzymes.

Solution	Concentration
DNA (plasmid or insert)	0.1-3 µg
NdeI (2U/µl)	2U/µg of DNA
SacI (2U/µl)	2U/µg of DNA
1:10 diluted BSA	5 µl
10X CutSmart buffer	5 µl
RNase/DNase-free water	to a final volume of 50 µl

2.4.4. Antarctic phosphatase digestion

Linearized plasmids were digested with Antarctic Phosphatase enzyme in order to remove the 5' phosphate group and prevent the plasmid self-ligation. The reaction was carried out in 0.5 ml eppendorf tubes (Table 2.9). Samples prepared as described in table 2.9 were incubated at 37 ºC for 1 hour followed by enzyme inactivation at 65 ºC for 20 minutes. After the Antarctic Phospatase enzymatic digestion, plasmids were purified as described in section 2.4.2 and quantified as previously described in section 2.3.1.

2.4.5. Ligation of plasmid and inserts

Ligation of pET28b+ plasmids and PCR products was performed using the T4 DNA ligase enzyme (PROMEGA). The amount of plasmid and insert DNA used in each reaction was calculated using the following equation:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

Following the T4 ligase supplier instructions (NEB), three ligation reactions were performed using plasmid-insert molar ratios of 1:1, 1:3 and 3:1, as the optimal molar ratio between plasmid and insert can vary depending on the size of the insert. Plasmid concentration was kept constant to 100 ng, with variation of the insert concentrations only (Table 2.10). A negative control, representing the reaction without insert, was used for each gene cloned. Samples were incubated at room temperature for three hours and then used for the bacterial transformation (section 2.4.7).

Table 2.9 Antarctic Phospatase reaction details.
Table showing the reactions components and their corresponding concentrations used for the Antarctic Phosphatase enzymatic reaction performed to remove the 5' phosphate group from the linearized plasmids. All reactions were performed in a final volume of 50 µl in 0.5 ml Eppendorf tubes.

Solution	Concentration
Plasmid DNA	0.1-3 µg
Antarctic Phosphatase (5U/µl)	5U/µg of DNA
1:10 diluted BSA	5 µl
10X Antarctic Phosphatase buffer	5 µl
RNase/DNase-free water	to a final volume of 50 µl

Table 2.10 Plasmid and insert ligation reaction details.
Table showing the ligation components with relative concentrations used. Three reactions were performed for each gene using the insert-plasmid molar ratios of 1:1, 1:3 and 3:1. Plasmid concentration was kept to 100 ng and the insert concentrations (X, 1/3·X and 3·X) were calculated using the equation described in section 2.4.5. All reactions were performed in a final volume of 10 µl.

Solution	1:1 (insert:plasmid)	1:3 (insert:plasmid)	3:1 (insert:plasmid)
Plasmid DNA	100 ng	100 ng	100 ng
Insert DNA	X	1/3·X	3·X
T4 ligase enzyme (1U/µl)	1 µl	1 µl	1 µl
10X Ligase buffer	1 µl	1 µl	1 µl
RNase/DNase-free water	to a final volume of 10 µl	to a final volume of 10 µl	to a final volume of 10 µl

2.4.6. Preparation of *E. coli* chemically competent cells

Before carrying out bacterial transformation with the ligated samples, competent *E. coli* JM109ES and BL21 cells were prepared. The protocol described here was kindly contributed by Andrew Spiers (Department of Plant Sciences, University of Oxford).

Overnight cultures of *E. coli* (JM109ES and BL21) were prepared as previously described (section 2.2). Fresh LB broth was inoculated with 1% of overnight culture and incubated at 37°C and 180 rpm shaking to an OD_{600} of 0.6. Cells were then harvested by centrifugation at 4500g for five minutes. After removing the supernatant, the pellet, containing all bacterial cells, was resuspended in 0.1 volumes (of the bacterial culture) of ice-cold 50 mM $CaCl_2$ and incubated on ice for 30 minutes. Cells were harvested again at 4500g for five minutes. The pellet was resuspended in 0.05 volumes of ice-cold 50 mM $CaCl_2$ and incubated on ice overnight. After the overnight incubation, Glycerol was added to a final concentration of 15% (v/v) and 200 µl aliquots were prepared in 1.5 ml eppendorf tubes and stored at -80°C.

2.4.7. Transformation of *E. coli* chemically competent cells

Ligation products obtained for each gene cloned (pET28b-*pa0949*, pET28b-*pa1204*, pET28b-*pa2280*, pET28b-*pa2580* and pET28b-*pa4975*), positive (empty pET28b plasmid) and negative (ligation reaction with no PCR product) controls were transformed into *E. coli* JM109ES cells using the heat shock method.

The chemically competent JM109ES cells were thawed on ice and five µl of ligation product were added. The tubes were then incubated for five minutes on ice, 45 seconds at 42° C and five minutes on ice again. Eight hundred µl of fresh LB broth were added to the cells and incubated at 37°C for an hour without shaking. The tube content was transferred on LB agar plates containing 30 µg/ml of kanamycin and incubated upside down overnight at 37°C.

After the clone screening via PCR and sequencing (section 2.4.8), plasmid containing the gene of interest were extracted as described in section 2.4.2 and used to transform *E. coli* BL21(DE3)pLysS cells (strain used for recombinant protein production) as described in this section.

2.4.8. Screening of positive clones

Twenty discrete colonies for each gene cloned were isolated from the LB agar plates inoculated with transformed *E. coli* cells. These were used to inoculate five ml of LB broth containing 30 µg/ml Kanamycin and incubated overnight at 37°C and 180 rpm shaking. Glycerol stocks were generated for each culture as previously described (section 2.2.1).

For each gene cloned five cultures were analysed via PCR in order to check the presence of the desired gene inserted. The colony PCR assay was performed using five µl of overnight cultures directly into the PCR reaction mix. Gene specific primers were designed to overlap part of the inserted gene sequence and part of the plasmid sequence resulting in a high specific pair of primers (Table 2.11). These were designed and analysed as previously described in section 2.3.2. The PCR reaction mix and thermocycling conditions are shown in table 2.12 and 2.13 respectively. PCR reactions using *E. coli* cells transformed with empty plasmid (representing the negative control) were also performed with each pair of primers.

The PCR products were then analysed on 1% (w/v) agarose gel as previously described (section 2.3.3). Five positive clones for each gene were grown as described in section 2.2. On these, plasmid extraction and quantification were performed as described in section 2.4.2 and 2.3.1 respectively. Two primers (Table 2.14) were designed on the pET28b+ plasmid sequence as described in section 2.3.2. The pET primers as well as plasmid samples for each gene cloned were sequenced as described in section 2.3.4.

Table 2.11 Primer used for colony PCR performed on single colony cultures of clones. Table showing the primers used for the screening of positive clones and their corresponding sequences.

Primer	Sequence (5'-3')	Target gene	Expected PCR product (bp)
CLpa0949 Fwd	GAGTCCTGGAGATCCTCATATG AGCAGTCCCTACATCCTG	*pa0949*	590
CLpa0949 Rev	GTGAGCTCTTGCGGGCCATTTC AACTCCCCAGCTTG		
CLpa1204 Fwd	CGAGGAGCACCATATGAGCGA CGACATCAAGGTATTG	*pa1204*	560
CLpa1204 Rev	CATGTTCGAGCTCTCCGGTGAC GAGGCATTCAAC		
CLpa2280 Fwd	CCGGAGGACATATGTCCGAAC AACTACCCAACCTCG	*pa2280*	690
CLpa2280 Rev	GGCGGACGGAGCTCAGAGCGA ACGCTGGTCGACCC		
CLpa2580 Fwd	CCAGCATGTACGGAACCATAT GAAAATCATTCTCCTGC	*pa2580*	590
CLpa2580 Rev	GGCCCGAGCTCGAACTCAGCC GGCGCG		
CLpa4975 Fwd	CGGAAACCCATATGAACGTAC TGATCGTCCACGC	*pa4975*	705
CLpa4975 Rev	CGCCGAGCTCAGCGCGCCAGC GGCTGG		

Table 2.12 Colony PCR reaction mix.
Table showing the PCR reaction mix components and their corresponding concentrations used for the screening of positive putative azoreductase gene cloned. Specific primers were used for each gene cloned as well as for the negative control. All reactions were performed in 25 µL final volume.

Solution	Volume (µl)	Final concentration
Bacterial culture	5	-
Forward primer (10 µM)	2.5	1 µM
Reverse primer (10 µM)	2.5	1 µM
2X HotStarTaq Master Mix (0.2 U/µl Taq polymerase, 3 mM $MgCl_2$, 400 µM dNTPs)	12.5	1X
DMSO	3	12%
RNase-free water	4.5 µl	-

Table 2.13 PCR Thermocycling condition used for the screening of positive clones.
Table showing the thermocycling steps and their duration times used for the PCR reactions performed to screen positive putative azoreductase gene cloned.

PCR step	Temperature (°C)	Time	N° of cycles
Taq polymerase activation	**95**	15 min.	1
Primers annealing	**60**	30 sec.	35
Fragments extention	**72**	1 min.	
DNA denaturation	**95**	30 sec.	
Primer annealing	**60**	30 sec.	1
Final extention	**72**	10 min.	1

Table 2.14 Primers used sequencing of positive clones.
Primers used to sequence the five putative azoreductase genes cloned. Forward (Fwd) and reverse (Rev) primers were designed both on the pET28b(+) sequence 50 bp upstream and 50 bp downstream of the gene sequence allowing optimum sequence reading.

Primer	Sequence (5'-3')	Target
pET28bseq Fwd	CATCATCACAGCAGCGGCCTGGTGC	pET28b 50 bp upstream start codon
pET28bseq Rev	GGCTTTGTTAGCAGCCGGATCTCAGTGG	pET28b 50 bp downstream start codon

2.5. Production of putative *P. aeruginosa* PAO1 azoreductase recombinant proteins

In order to express the putative azoreductase proteins, pET28b+ vector containing each of these genes were extracted from *E. coli* JM109ES and transformed into *E. coli* BL21 competent cells and screened via PCR as previously described (section 2.4.7).

All recombinant proteins were produced first at small scale in order to find the optimum condition for the large scale production. Proteins were produced following a similar protocol described for paAzoR1-3 (Wang et al., 2007, Ryan et al., 2010a). However, modifications of the original protocol were applied and they are described here.

2.5.1. Small scale recombinant protein production

E. coli BL21 transformed with pET28b-insert, representing the expression samples (pET28b-*pa0949*, pET28b-*pa1204*, pET28b-*pa2280* and pET28b-*pa2580*), and with empty pET28b (empty plasmid representing the negative control) were grown over night in LB broth adding 30 μg/ml kanamycin. Five hundred μl of overnight culture were used to inoculate 50 ml of LB containing 1M sorbitol, 30 μg/ml kanamycin, and 2.5 mM betaine in 200 ml flasks. These were incubated at 25°C and 180 rpm shaking to an OD_{600} of 0.5. 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the recombinant protein production for 16 hours at 18°C and 180 rpm shaking. After induction, the cultures were centrifuged at 4500g for 20 minutes using the 5804R centrifuge (Eppendorf), the supernatant was removed and the pellet was resuspend with 1 ml of lysis buffer (20 mM TrisHCl, 100mM NaCl, 1 protease inhibitor tablet, 0.02 mg/ml DNase and 0.2 mg/ml lysozyme, pH 8). The samples were then freeze-thawed four times using liquid nitrogen and a 37° C water bath. A sample, representing the whole cell lysate was collected for further protein gel analysis. The cell lysate was centrifuged at 16000g for 20 min at 4°C to allow the separation of the soluble fraction (supernatant) from the insoluble (pellet).

To make a direct comparison between fractions, the insoluble was resuspended with 1 ml of 20 mM TrisHCl 100mM NaCl buffer, representing the same volume of lyses buffer used and which represents the soluble fraction volume after its separation. Soluble and insoluble fractions were collected and analysed, together with the whole cell lysate on SDS-PAGE (section 2.5.3). Protein molecular weights were determined as described in section 2.3.3.

2.5.2. Large scale recombinant protein production

Following the results obtained with the small scale protein expression, larger culture volumes were used to produce the amount of proteins necessary for further analysis using expression samples and negative controls (see section 2.5.1). To do this, all expression conditions were kept the same as those used for the small scale expression, except for the changes explained below.

Five hundred ml culture was used to produce each recombinant protein following the procedure described in the previous section, (kanamycin, betaine, sorbitol and IPTG concentrations, temperatures and time of incubation and centrifugation). After the separation of the pellet, this was resuspend with 20 ml of lysis buffer (20mM TrisHCl, 100mM NaCl, 1 protease inhibitor tablet (Roche), 0.02 mg/ml DNase and 0.2 mg/ml lysozyme, pH 8). The samples were then kept on ice and sonicated using the MSE Soniprep 150 (30 cycle, 45 sec on and 30 sec off). The cell lysate was centrifuged at 16000g for 20 min at 4°C to allowing the separation of the soluble and insoluble fractions using the Sorvall RC6+ centrifuge (Thermo Scientific). The soluble fraction was removed and the insoluble was resuspended with 20 ml of 20 mM TrisHCl 100 mM NaCl buffer. Whole cell lysate, soluble and insoluble fractions were collected and analysed on SDS-PAGE (section 2.5.3). Protein molecular weights were determined as described in section 2.3.3.

2.5.3. SDS-PAGE analysis of recombinant proteins

The fractions collected (whole cell lysate, soluble and insoluble) for both, expression samples (pET28b+ containing putative azoreductase genes) and negative control (empty pET28b+) were analysed by SDS-PAGE. The gel consisted of a resolving gel (12% acrylamide:bis acrylamide (29:1), 0.125% w/v SDS, 0.05 w/v ammonium persulfate (APS), 0.002% v/v N',N',N',N'-tetramethylethylenediamine (TEMED) in 0.375 M TrisHCl pH 8) and a stacking gel (6% acrylamide:bis acrylamide (29:1), 0.125% w/v SDS, 0.05% w/v APS, 0.002% v/v TEMED in 0.125 M TrisHCl pH 6.8). Sample and control fractions were prepared adding 1:5 gel loading dye (40% w/v sucrose, 0.25% w/v bromophenol blue and 5 mg/mL DTT in H_2O). These were then incubated at 95°C for 5 minutes for the complete protein denaturation.

Ten µl of each fraction for both, expression sample and negative control, were loaded on the gel and Amersham ECL High-Range Rainbow Molecular Weight Marker (GE Healthcare Life Sciences) was used as standard. Gels were run in the MiniProtean Tetra system (BIO-RAD) applying 180 volts for 45 minutes and using 0.25 M Tris, 1.95 M Glycine and 1% SDS running buffer (National Diagnostic). The gels were stained for 40 minutes in comassie stain solution (0.0024% w/v comassie brilliant blue, 50% v/v methanol/IMS, 10% v/v acetic acid, 40% v/v H_2O) and distained overnight in a distain solution (10% v/v ethanol, 10% v/v acetic and 80% v/v H_2O). Gels were visualised as described in section 2.3.3.

2.6. Purification of putative *P. aeruginosa* PAO1 azoreductase recombinant proteins

The recombinant proteins PA0949, PA1204, PA2280 and PA2580 produced (section 2.5) were purified as described for paAzoR1-3 (Wang et al., 2007, Ryan et al., 2010a). However, modifications of the original protocol were applied and they are described here.

2.6.1. Immobilised Metal-ion Affinity Chromatography (IMAC)

To purify the produced recombinant proteins, the Immobilised Metal-ion Affinity Chromatography (IMAC) method was used. For this process Ni-NTA agarose matrix (Invitrogen) was used to bind the hexahistidine residues present on each protein.

Four ml of Ni-NTA beads in 20% (v/v) ethanol were loaded into a 20 ml chromatography columns (with a porous polymer bed support at the bottom of the column), the ethanol was removed and two washes with 20 mM TrisHCl 100 mM NaCl pH 8 buffer were performed to remove any ethanol residues.

Soluble fraction (20 ml) was then applied to the column and mixed well with the beads by inverting the column 4-5 times. The column was incubated for 10 minutes on ice to allow the protein to bind the nickel before proceeding. The liquid phase was removed applying pressure using a 10 ml syringe until the solution reached the same level of the beads, avoiding to drying out completely the matrix. This fraction was collected as flow through.

Increasing concentrations of imidazole (10, 25, 50, 100, 250 and 500 mM) in 20 mM TrisHCl 100 mM NaCl buffer were prepared and used to elute the proteins. All eluates were collected and analysed on SDS-PAGE as previously described (section 2.5.3). Protein molecular weights were determined as described in section 2.3.3.

2.6.2. Buffer exchange dialysis

To remove the excess of imidazole, the elution containing the recombinant proteins were placed into a molecular porous membrane tubing (SPECTRUM®, Spectrum Laboratories Inc.) with molecular weight cut-off of 12-14 KDa. The tubes were placed into a four litres dialysis buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8) overnight at 4° C with magnetic stirring agitation.

2.6.3. Hexahistidine tag detection

In order to detect the presence of the His tag on each of the recombinant proteins purified, western blot analysis was carried out. Three samples of each purified recombinant protein (5, 1 and 0.2 μg) were run on SDS-PAGE as described in section 2.5.3. The protein bands were transferred on Amersham Hybond-P 0.45 PVDF membrane (GE Healthcare) using the TE77 ECL Semi-dry transfer unit (GE Healthcare). In particular, the membrane was pre-wet with methanol for 10 seconds and washed in transfer buffer (Tris-HCl 48mM, SDS 0.037% (w/v), Glycine 39mM, Methanol 20% (v/v), pH 8.3). Three pre-wet (with transfer buffer) Grade 3MM Chr blotting paper (GE Healthcare) were placed onto the transfer unit. On the top on these the membrane, the gel and other three blotting paper were placed. The transfer unit was set to 43 mA per gel used for two hours.

After the transfer, membranes were blocked using low fat milk powder 5% (w/v) in TBS buffer (TBS buffer (Tris-HCl 20mM, NaCl 137 mM, pH 7.6) containing 0.1% Tween-20 (v/v)) for 2 hours. 1:1000 diluted His-probe Antibody (H-15) (Santa Cruz Biotechnologies) was prepared in TBS-Tween buffer containing 1% milk and this was used to probe the membranes overnight at 4° C. The membranes were then washed using TBS-Tween buffer changing the buffer every 20 minutes for two hours. 1:1000 diluted HRP-conjugated donkey anti-rabbit (NA934V) secondary antibody (GE Healthcare) was prepared in TBS-Tween buffer containing 1% milk and used to probe the membranes for 1

hour at room temperature. Washes with TBS-Tween buffer were repeated every 15 minutes for four times (one hour).

For the detection step Amersham ECL western blotting detection reagents (GE Healthcare, UK) were used directly on the membranes and sealed with a transparent film in a cassette. Scientific imaging films were exposed to the membrane into the cassette for different times (until detectable bands were visible) and developed using FUJI Medical film processor RG II. The blots were visualized using Model GS-800 Calibrated Imaging Densitometer.

2.6.4. Removal of Hexahistidine Tag

To remove the histidine residues present on the N-terminus of each protein, an enzymatic reaction was carried out using thrombin. A thrombin stock was prepared to 1 U/µl and 1 U of enzyme per mg of protein was used in each reaction. After mixing protein and enzyme, the reaction was incubated for 16 hours at 4° C.

To verify the completion of the hexahistidine tag cleavage, digested and undigested samples were compared on SDS-PAGE as previously described (section 2.5.3).

2.6.5. Protein quantification and concentration

After the overnight dialysis, the proteins were quantified by reading the absorbance at 280 nm using a biophotometer (Eppendorf) and converting the values in mg/ml using the Beer-Lambert's Law:

$$A = \varepsilon C L$$

Where A is the absorbace, ε is the extinction coefficient, C is the protein concentration and L is the path length. The ε values (units of M^{-1} cm^{-1}) were calculated for each protein using ExPASy ProtParam tool in which the full amino acid sequence, including the histidine

residues, were inserted for each protein (Gasteiger et al., 2005). The extinction coefficient values were then divided for the protein molecular weight giving the Ɛ values to use in the Beer-Lambert's Law. Found Ɛ and considering that L=1 cm, the concentration of each protein was calculated in g/l and then converted in mg/ml.

All proteins were then concentrated to 2 mg/ml using 10 KDa cut off 50 ml spin columns (Viva Science) following the supplier instruction and stored at -80°C in 5% glycerol.

2.7. Characterization of putative *P. aeruginosa* PAO1 recombinant azoreductase proteins

The expressed and purified recombinant proteins PA0949, PA1204, PA2280 and PA2580 were characterized performing a series of analysis including the presence and determination of flavin group, NAD(P)H and flavin preference during the enzymatic reaction and substrate specificity using azo- and quinone- substrates.

2.7.1. Flavin group determination

2.7.1.1. Protein spectra

To detect the presence of the flavin, absorpition spectra were generated for each protein. One mg/ml protein solutions were prepared diluting the 2 mg/ml stocks (section 2.6.2) with 20 mM tris 100 mM NaCl pH 8 buffer and compared with 10 and 20 μM flavin solutions prepared in the same buffer. Three spectra for each solution (protein, FMN and FAD) were generated (Table 2.15) using: i) only protein or flavin solutions; ii) adding 1 mM DTT; iii) heating the solutions + DTT at 95°C for 5 minutes. One ml of solution was placed in UV transparent cuvettes and all spectra were generated using the Infinite M200 PRO plate reader (TECAN). Absorbance measurements were taken every 2 nm from 300 to 600 nm.

Table 2.15 Absorption spectra analysis conditions of recombinant protein and flavin solutions.

Table showing the recombinant protein and flavin solutions used to generate absorbance spectra. These were generated on the pure protein or flavin solutions, after adding DTT and after heating the pure solutions plus DTT.

Spectrum	Solution (protein or FMN or FAD)	DDT	Heating (95° C)
1	+	-	-
2	+	+	-
3	+	+	+

2.7.1.2. Thin Layer Chromatography

To verify the presence and the kind of the flavin co-factor present in each protein, Thin Layer Chromatography (TLC) assays were carried out using pure recombinant proteins, FMN and FAD as described by Liu and colleagues (Liu et al., 2008).

Free FMN and FAD were prepared in 20 mM TrisHCl 100 mM NaCl pH 8 buffer. The assays were performed using silica gel coated glass plates and N-butanol, acetic acid and water (2:1:1 ratio) as solvent. The assays were optimized using different flavin concentration (0.5, 1, 5 and 10 mM) and 0.5 mM was chosen. One µl of 0.5 mM FMN and FAD and 1 µl of 20 mg/ml protein (prepared from 2 mg/ml protein stock concentration) were spotted on the plate. This was placed into a beaker containing the solvent and covered with parafilm. The solvent was allowed to migrate up to one cm before the edge of the plates.

Retention factor (R_f) values were determinate by dividing the distance travelled by the compound by the total distance travelled by the solvent. Pictures were taken using the Molecular Imager Gel Doc™ XR+gel (BIO-RAD).

2.7.2. Enzymatic assays

2.7.2.1. Calculation of molar extinction coefficient

Absorption spectra of azo compounds as well as NAD(P)H were generated prior assessing the enzymatic activity of recombinant proteins. These were generated using one ml of 50 µM solution in 20 mM TrisHCl 100 mM NaCl pH 8 buffer. The solutions were placed in UV transparent cuvettes and all spectra were generated as described in section 2.7.1.

The maximum wavelengths obtained for azo compounds and for NAD(P)H were used to calculate the molar extinction coefficient (ε), a parameter needed for the calculation of the reduction rate of each enzyme. To do this, the absorbance of dilutions of

substrates and NAD(P)H (500, 300, 200, 100, 10 and 0 μM) were measured using their maximum absorption wavelength as described in section 2.7.1.1. Regression analysis was then performed on the obtained absorbance values and ε were obtained.

2.7.2.2. NAD(P)H and flavin preference determination

To verify the NAD(P)H and flavin preference during the enzymatic reduction of recombinant azoreductase proteins, a series of enzymatic assays were performed using some azo- end quinone- substrates. In some cases, quinones were used as no or low activity with azo substrates was observed.

To monitor the enzymatic activity, specific absorptions were followed for azo compounds (Table 4.2), whereas for the quinones the NAD(P)H oxidation was followed at 340 nm as indication of substrate reduction. The assays were carried out in 96 well plates and absorbance was measured every 15 seconds for 30 minutes using the Infinite M200 PRO plate reader. The reaction mix used is shown in table 2.16.

To determine the NAD(P)H and flavin preference, a series of combinations were used (Table 4.3 and 4.4) and reduction rates were calculated for each of these. Reduction rates were obtained using the Last Square method for the first 300 second of reaction (when the reductions show a linear profile). This was then divided for the molar extinction coefficient and then for the amount of protein used (in mg/ml) in order to obtain μM of substrate reduced per second per mg of protein ($\mu M \cdot s^{-1} \cdot mg^{-1}$):

$$\mu M \cdot s^{-1} \cdot mg^{-1} = (\text{linear regression} / \varepsilon) / \text{mg of protein}$$

Table 2.16 Reaction mix used for the NAD(P)H and flavin determination enzymatic assays.

Table showing the reaction mix used for the determination of NAD(P)H and flavin preference during the enzymatic reaction of putative P. aeruginosa PAO1 recombinant proteins.

Solution	Final concentration	Volume (µl)
Substrate	50 µM	5
NADH or NADPH	500 µM	5
FMN or FAD	1 µM	5
Protein	10 µg	5
Buffer (20 mM TrisHCl and 100 mM NaCl pH 8)	-	80

2.7.2.3. Substrate specificity

In order to characterize the activity of each recombinant protein with azo- and quinone- substrates (Table 2.17 and APPENDIX V), a series of enzymatic assays were carried out.

Different protein concentrations were used to test azo compounds (0-200 μg/ml) and quinones (0-50 μg/ml). Azocompound and NAD(P)H absorbance maximums (λ_{max}) were determined as described in section 2.7.2.2. The λ_{max} of each compound was used to measure the decreasing in absorbance due to the compound reduction. For the quinones, the oxidation of NAD(P)H was followed at 340 nm as indication of the substrate reduction.

The enzymatic reactions were prepared as described in table 2.16. Each enzyme concentration was loaded in 96 well plate in triplicate. All other reaction components were mixed well in the appropriate proportion and added to the 96 well plate prior to start the assay (Table 2.16). Absorbance was monitored every 15 seconds for 30 minutes using the Infinite M200 PRO plate reader (TECAN), and determination of substrate specificity was carried out as described in section 2.7.3.2.

Table 2.17 Substrates used for the *P. aeruginosa* PAO1 putative azoreductase proteins characterization.

Table showing the substrates used for the enzymatic characterization of the putative azoreductase protein. A: azocompound; Q: quinone. Structure of these substrates are shown in APPENDIX V.

Name/abbreviation	Compound	Class
Amaranth	1-(4-sulfo-1-naphthylazo)-2-naphthol-3,6-disulfonic acid trisodium salt	A
Balsalazinde	5-[4-(2-Carboxyethylcarbamoyl)phenylazo]salicylic acid disodium salt	A
Methyl red	2-(4-Dimethylaminophenylazo)benzoic acid	A
Orange G	1-Phenylazo-2-naphthol-6,8-disulfonic acid disodium salt	A
Orange II	4-[(2-Hydroxy-1-naphthalenyl)azo]benzenesulfonic Acid Monosodium Salt	A
Ponceau S	3-Hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]phenylazo)-2,7-naphthalenedisulfonic acid sodium salt	A
Ponceau BS	disodium 2-[(2-hydroxy-1-naphthyl)azo]-5-(4-sulfonatophenyl)azo-benzenesulfonate	A
Sudan I	1-Phenylazo-2-naphthol	A
Tropaeolin	4-([2,4-Dihydroxyphenyl]azo)benzenesulfonic acid	A
ANI	*N,N*-dimethylindoaniline	Q
AQS	sodium anthraquinone-2-sulfonate	Q
BZQ	1,4-benzoquinone	Q
DCB	2,5-dichloro-1,4-benzoquinone	Q
HNQ	5-hydroxy-1,4-naphthoquinone	Q
IBC	2,6-dichloroquinone-4-chloroimide	Q
LAW	2-hydroxy-1,4-naphthoquinone	Q
MEN	2-methyl-1,4-naphthoquinone	Q
ONO	1,2-naphthoquinone	Q
PLU	5-hydroxy-2-methyl-1,4-naphthoquinone	Q

2.8. Morphological characterization of *P. aeruginosa* PAO1 azo- and quinone oxidoreductase gene transposon mutants

2.8.1. Generation of stocks of *P. aeruginosa* PAO1 wild type and transposon mutants

P. aeruginosa PAO1 wild type and transposon mutants for the genes *paazor1*, *paazor2*, *paazor3*, *pa2580*, *pa2280*, *pa1204*, *pa0949* and *pa4975* were received in stab cultures on LB agar. Each culture was used to inoculate 9 mm petri dishes containing LB agar for wild type and LB agar with 12.5 μg/ml of tetracycline for all mutants. All plates were incubated upside down overnight at 37°C in a static incubator. Single colonies were isolated for each strain and used to generate glycerol stocks as previously described (section 2.2.1).

2.8.2. Assessment of *P. aeruginosa* PAO1 transposon mutants

In order to check the transposon insertion for each mutant, PCR amplification was performed. The genes *paazor1*, *paazor2*, *paazor3*, *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*, which were used as target for the amplification, are made of no more than 700 nucleotides (0.7 Kb). One of the transposon mutants feature is to have the ISphoA/hah (5 Kb) or ISlacZ/hah (6.7 Kb) sequence inserted within the sequence coding region of the mutated gene (Figure 2.1) (Jacobs et al., 2003). Considering that the DNA polymerase used is able to amplify one Kb of DNA per minute, one minute was used as extension time. This allowed the amplification of the genes without any insertion (in wild type), but not in the transposon mutants.

The *P. aeruginosa* PAO1 wild type and transposon mutant stocks generated from single colony cultures were used to generate bacterial liquid cultures as previously described (section 2.2). For each culture, genomic DNA extraction and quantification were performed as described in section 2.3.1.

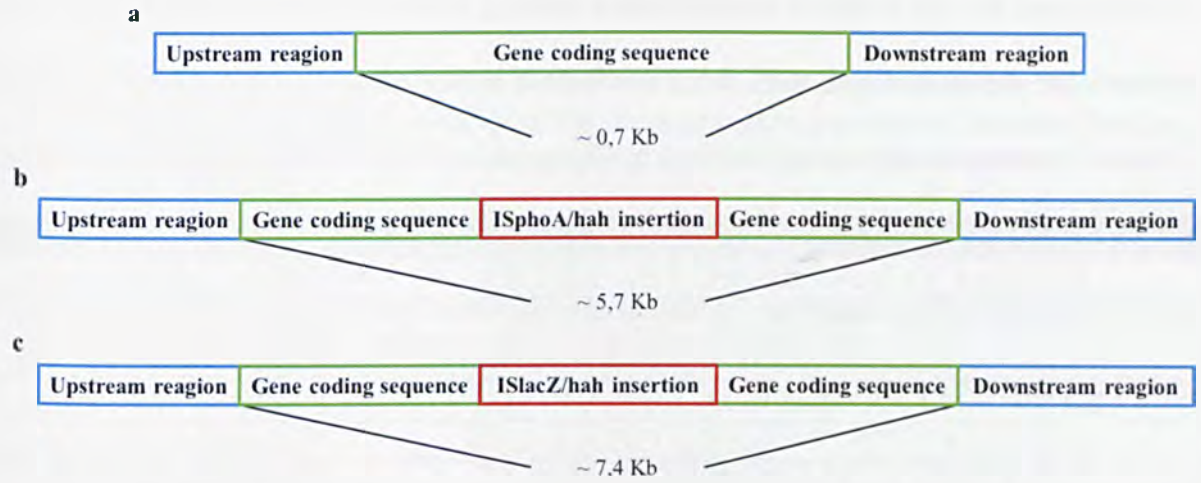


Figure 2.1 Repesentation of ISphoA/hah and ISlacZ/hah transposon insertion within the gene coding sequences.
Figure showing the genetic representation of transposon mutations in *P. aeruginosa* PAO1. In the wild type strain, the gene coding sequence is flanked by upstream and downstream non-coding regions and it is made of approximately 0.7 Kb (**a**). When transposon insertions are present in the middle of the coding sequence, the region between the non-coding sequence results in approximately 5.7 Kb or 7.4 Kb, as a consequence of the insertion of ISphoA/hah (**b**) and ISlacZ/hah (**c**) respectively.

PCR amplification of target genes was performed using the gene specific primers already used for the putative azoreductases cloning (section 2.4.1 and Table 2.7). The primers for the amplification of the azoreductase genes *paazor1*, *paazor2* and *paazor3* (Table 2.18) were designed as described by Wang and colleagues and Ryan and colleagues (Wang et al., 2007, Ryan et al., 2010b). All primers used overlap part of the coding and part of the non-coding sequence, obtaining high binding affinity and specificity only with the desire nucleotide region.

PCR reaction mixes were prepared using the HotStar Taq DNA Polymerase Kit following the supplier instruction. The table 2.19 shows the PCR reaction mix components and the volume of each component used to perform the reaction.

The PCR thermocycling conditions were optimized using different annealing temperatures to find the optimum for all genes to amplify and they are shown in table 2.20. The PCR products were analysed on 1% (w/v) agarose gel as previously described (section 2.3.3).

2.8.3. Growth analysis of *P. aeruginosa* PAO1 wild type and transposon mutants

P. aeruginosa PAO1 wild type and transposon mutants growth was tested in LB broth (media details in table 2.2, section 2.2). The assays were performed in 96 well plates using 95 µl of appropriate medium inoculated with five µl of bacterial overnight culture to a final OD_{600} of 0.05 in a total volume of 100 µl. Plates were sealed with sterile thermal adhesive sealing films (Thermo Scientific) and incubated at 37° C in the Infinite M200 PRO plate reader (TECAN) for 20 hours. OD_{600} was measured every 30 minutes with 5 seconds plate shaking before every measurement. For each strain three replicates were used.

Table 2.18 Gene specific primers for the known azoreductase genes *paazor1-3*.
Table showing the primer sequences used for the single gene transposon mutants checking for the *P. aeruginosa* PAO1 azorereductase genes *paazor1*, *paazor2* and *paazor3*.

Primer	Sequence (5'-3')
paazor1 Fwd (*paazoR1*)	CATTCGAGTCTAGCCGAATCCAAGGAG
paazor1 Rev (*paazoR1*)	CACCAGCAGATGGAACCAAGGCCATG
paazor2 Fwd (*paazoR2*)	GATGCAAACCACTCGATCGCCAACCACTG
paazor2 Rev (*paazoR2*)	GATGCAAACCACTCGATCGCCAACACTG
paazor3 Fwd (*paazoR3*)	GATACATATGTCCCGTGTCCTGGTTATCG
paazor3 Rev (*paazoR3*)	CCCGGCCGTCACACCGCAACCAT

Table 2.19 PCR reaction mix used for the single gene transposon mutants checking.
Table showing the PCR reaction mix components and their corresponding concentrations used for the single gene transposon mutants checking. Specific primers were used for each transposon mutant. All reactions were performed in 25 µl final volume.

Solution	Volume (µl)	Final concentration
Genomic DNA	1	100 ng
Forward primer	2.5	1 µM
Reverse primer	2.5	1 µM
HotStarTaq Master Mix	12.5	-
DMSO	3	12%
RNase-free water	To a final volume of 25 µL	-

Table 2.20 PCR thermocycling condition used for the mutants checking.
Table showing the thermocycling steps and their corresponding duration times used for the PCR reactions performed to single gene transposon mutant checking.

Step	Temperature (°C)	Time	N° of cycles
Initial denaturation	95	15 min.	1
Annealing	55	30 sec.	35
Extension	72	1 min.	
Denaturation	95	30 sec.	
Annealing	55	30 sec.	1
Final exention	72	10 min.	1

2.8.4. Motility in *P. aeruginosa* PAO1 wild type and transposon mutants

Motility of *P. aeruginosa* PAO1 wild type and transposon mutant strains was assessed a similar method previously published (Rashid and Kornberg, 2000). Bacterial overnight cultures were generated for all strains as previously described (section 2.2). Five µl aliquots of each bacterial overnight culture were inoculated onto the surface of LB, M9 and BHI agar (0.5% w/v Bacto agar (Difco) in LB broth, M9 broth and BHI broth media) and incubated overnight at 37°C. Three replicates were spotted in each plate.

The ability to swarm was assessed by measuring the distance of swarming from the central inoculation site to the edge of the colony.

2.8.5. Biofilm formation by *P. aeruginosa* PAO1 wild type and transposon mutants

In order to analyse the effect of mutating azoreductase genes on the *P. aeruginosa* biofilm formation a series of experiments were performed to establish a robust method. These were performed first using the wild type strain only and then all the transposon mutants as well, following a similar protocol described by Merritt and colleagues (Merritt et al., 2005). Two different methods, falcon tubes and 96 well plates, were tested and they are described here.

2.8.5.1. Falcon tube method

P. aeruginosa PAO1 wild type strain was growth overnight and used to inoculate ten ml of LB broth to an OD_{600} of 0.2. Sterile microscope cover slips were placed into the 50 ml tubes containing the inoculated medium and used as surface for biofilm attachment. The tubes were incubated at 37° C in a static incubator for four hours and samples were collected and analysed every hour.

Cover slips were taken from each falcon tube and incubated in five ml of 1% (v/v) Crystal Violet (CV) solution for 15 minutes in 9 cm petri dishes. Cover slips were then

washed in distilled water by immersion and allowed to air dry before incubating them in five ml of 95% (v/v) ethanol for 15 minutes on a shaker at 100 rpm. A one hundred µl aliquot of destain solution (95% v/v ethanol) was transferred to a clean 96 well plate, and the OD_{540} was measured using the Infinite M200 PRO plate reader (TECAN). Samples were used in triplicate.

2.8.5.2. 96 well plate method

P. aeruginosa PAO1 wild type overnight cultures were generated as previously described (section 2.2). Ninety-five µl of LB broth medium were inoculated with five µl of bacterial overnight culture in a total volume of 100 µl in 96 well plates. Six starting OD_{600} were tested including 0.02, 0.05, 0.1, 0.2, 0.3, and 0.4. The plates were sealed with thermal adhesive sealing films and statically incubated at 37° C for 24 and 48 hours.

After the incubation, the plates were washed with distilled water by immersion to remove planktonic growth and allowed to air dry. Hundred and twenty-five µl of 1% (v/v) CV solution were added to the wells and incubated for 15 minutes. Plates were washed in distilled water by immersion and allowed to air dry. Two hundred µl of 95% (v/v) ethanol was added to each well and left for 15 minutes on a shaker at 100 rpm. One hundred µl were was transferred to a clean 96 well plate, and the OD_{540} was measured using the Infinite M200 PRO plate reader (TECAN).

The biofilm assay for *P. aeruginosa* PAO1 wild type and transposon mutant strains was then conducted using the starting OD_{600} of 0.4 (which had produced the most significant biofilm for both 24 hours and 48 hours incubations) and the same procedure described above.

The biofilm production results were analysed by calculating the mean of the replicates and the standard error of the mean. From the raw data, the negative control value was subtracted, and the values were normalised to a path length ratio of 4. This was

experimentally determined by finding the ratio between the absorbance values from the 96 well plate and cuvette readings, in order to account for the different path lengths. This was done using a solution of 50 μM Methyl red. Results were statistically analysed by an unpaired t-test and two-way ANOVA accordingly using GraphPad Prism 6.

2.8.6. Antibiotic resistance of *P. aeruginosa* PAO1 wild type and transposon mutants

2.8.6.1. Minimal Inhibitory Concentration (MIC) analysis

To test the effect of mutating azoreductase and putative azoreductase genes on antibiotic resistance, MICs were determined as described by Coyle and colleagues (Coyle, 2005).

Overnight cultures were generated for wild type and transposon mutant strains as described in section 2.2. Mueller-Hinton broth medium was prepared adding one antibiotic each time to the highest antibiotic concentration to test. From this, using the microdilution method, six more antibiotic concentrations were obtained with doubling dilution (1:2) using the same medium. The same medium containing only the vehicle solution (solvent used to prepare the antibiotics, Table 2.21) and without antibiotic was used for the positive controls, in order to show the viability of each strain and the absence of any effect due to the solvent used to prepare each antibiotic.

Fourteen antibiotics (details shown in table 2.21) were tested on wild type and transposon mutant strains. The assays were performed in 96 well plates using 95 μl of appropriate medium inoculated with five μl of bacterial overnight culture to a final OD_{600} of 0.05 in a total volume of 100 μl. The plates were covered with thermal adhesive sealing films and incubated at 37° C for 20 hours. OD_{600} measurements were taken after 20 hours from the incubation using the Infinite M200 PRO plate reader (TECAN). Three replicates were used for each strain.

Table 2.21 Antibiotics used for the *P. aeruginosa* PAO1 wild type and single gene transposon mutants antibiotic resistance analysis.
Table showing the antimicrobial compounds used for MIC test of *P. aeruginosa* PAO1 wild type and single gene transposon mutants. Solvents used to prepare the antibiotic solutions as well as their concentrations used in the assays are also shown.

Antibiotic	Solvent	Concentration µg/ml	Supplier
Ceftazidime	PBS	0-32	TCI
Ciprofloxacin	0.1 M HCl	0-2	TCI
Chloramphenicol	EtOH	0-200	Sigma
Colistin	PBS	0-16	Sigma
Gentamicin	PBS	0-15	TCI
Levofloxacin	0.1 M HCl	0-8	TCI
Meropenem	DMSO	0-8	TCI
Nalidixic acid	0.1 M HCl	0-500	TCI
Norfloxacin	0.1 M HCl	0-16	TCI
Novobiocin	PBS	0-2000	Sigma
Piperacillin	PBS	0-8	TCI
Rifampicin	DMSO	0-50	Sigma
Tigecycline	EtOH	0-32	TCI
Tobromycin	PBS	0-15	Sigma

2.8.6.2. Minimal Bactericidal Concentration (MBC) analysis

For the antibiotics ciprofloxacin, levofloxacin, meropenem, tobromycin, colistin and tigecycline the MBCs for wild type and transposon mutant strains were determined using a similar protocol described by Andrews and colleagues(Andrews, 2001). Using these antibiotics MIC assay was performed.

Following the MIC determination, 50 µl of culture from wells corresponding to the positive control (no antibiotic), MIC and higher antibiotic concentrations (which didn't show a bacterial growth in the MIC determination) were used to inoculate LB agar plates containing 12.5 µg/ml tetracycline. The Plates were then incubated upside down overnight at 37° C.

Bacterial growth was visually detected and recorded with pictures. The lowest antibiotic concentration in which bacterial growth has not been shown on plates, was considered as MBC. Three replicates were used for each strain.

Chapter 3. Cloning and expression of putative azoreductases from *P. aeruginosa* PAO1

3.1. Introduction

P. aeruginosa is able to colonize a wide range of environmental habitats, including aquatic sediments, water exposed surfaces, soil, plant roots and leaves, human and animal sewage (Ringen and Drake, 1952, Green et al., 1974a, Pellett et al., 1983b). This extremely adaptable bacterium is able to infect a large number of different hosts including, humans, insects, nematodes and plants (Apidianakis and Rahme, 2009, Tan et al., 1999, Starkey and Rahme, 2009a). In order to understand the genetic basis of versatility and infectivity of this pathogen, a genome scale analysis approach is crucial.

The *P. aeruginosa* PAO1 genome was sequenced in 2000 using the whole-genome-shotgun approach (Stover et al., 2000). This made available a powerful new set of information for understanding the genetic bases of the ecological versatility and pathogenicity of *P. aeruginosa*.

The genome of *P. aeruginosa* PAO1 is approximately 6.3 Mb and comprises 5,570 predicted open reading frames (ORFs) more than 95% of which are highly conserved (Wolfgang et al., 2003). Analyses of this genome revealed a high proportion of regulatory genes (8.4%) which modulate the diverse genetic and biochemical properties of this bacterium to adapt to changing environmental conditions. There are also many genes responsible for growth and metabolism of various organic compounds which is likely to enhance the ability of the bacterium to survive under harsh environmental conditions (Stover et al., 2000).

Additionally, The Pseudomonas Genome Database, an user-friendly online database, was developed in 2001 to improve the use of the genetic data available and since then it has been a resource for peer-review, continually-updated annotations for the *P.*

aeruginosa PAO1 reference strain's genome and, more recently, comparative analyses of several related *Pseudomonas* species (Winsor et al., 2005, Winsor et al., 2009, Winsor et al., 2011).

The availability of the entire *P. aeruginosa* PAO1 genome sequence and the opportunity to perform comparative genomic analysis can lead to the identification of genes with putative functions. A good example of putative gene identification has been described for three azoreductase genes (*paazor1-3*) which were identified in *P. aeruginosa* PAO1 based on sequence homology to *E. coli* AzoR gene (Wang et al., 2007).

A more detailed approach to identify new putative azoreductase genes was used by Ryan and colleagues on the same bacterium. In the latter case the genes were identified by comparing protein sequence, function and structure of azoreductases from many different organisms to *P. aeruginosa* PAO1 proteins. (Ryan et al., 2014). This study proposed that there are seven additional genes in *P. aeruginosa* PAO1 which may show an azo- and/or quinone oxidoreductase enzymatic activity (Table 3.1).

Although the proteins encoded by these genes have been hypothesised to have similar enzymatic functions, their nucleic acid sequences show low homology, which are not greater than 37% (Table 3.2). This has also been observed comparing putative and known azoreductase genes in *P. aeruginosa* PAO1, where there is also low sequence homology (Table 3.2). Regarding the location in the genome, the putative azoreductase genes are located far from each other and as a consequence are unlikely to be co-regulated (Table 3.1). In addition, the genes *pa1224* and *pa1225*, which are adjacent on the chromosome, are unlikely to be co-regulated because they are on opposite strands. This information indicates the absence of any correlation between these genes as well as with the known azoreductase 1, 2 and 3. However, structural and functional data of homologous gene products from other organisms strongly suggests that these genes may encode for proteins which have an azo- and/or quinone oxidoreductase function.

Table 3.1 Putative *P. aeruginosa* PAO1 azoreductase gene details.
Table showing the name, location in the genome and size of seven putative azoreductase genes from *P. aeruginosa* PAO1.

	Gene name	Alternative name	Genome location (bp)	Size (bp)
pa0949	*wrba*	-	1036579-1037175	597
pa1204	-	*yieF*	1303892-1304449	558
pa1224	-	-	1327024-1327803	609
pa1225	-	-	1327813-1328439	642
pa2280	-	*arsH*	2508775-2509467	693
pa2580	-	*mdaB*	2916158-2916748	591
pa4975	-	-	5591452-5592612	705

Table 3.2 nucleic acid identity matrix between known and putative *P. aeruginosa* PAO1 azoreductase genes.
Table showing the nucleic acid sequence homology between known (*paazor1*, *paazor2* and *paazor3*) and putative (*pa0949*, *pa1204*, *pa1224*, *pa1225*, *pa2280*, *pa2580* and *pa4975*) azoreductase genes from *P. aeruginosa* PAO1. Values are expressed in percentage of homology. Sequences have been taken from Pseudomonas Database (Winsor et al., 2011) and identity matrix was generated on a ClustalW aligment using Bioedit software (Hall, 1999).

	paazor1	*paazor2*	*paazor3*	*pa2580*	*pa2280*	*pa1204*	*pa0949*	*pa4975*	*pa1224*	*pa1225*
paazor1	ID	55%	48%	30%	32%	36%	31%	36%	37%	37%
paazor2	55%	ID	50%	29%	33%	33%	31%	35%	33%	37%
paazor3	48%	50%	ID	28%	31%	32%	27%	36%	35%	37%
pa2580	30%	29%	28%	ID	25%	27%	28%	33%	28%	35%
pa2280	32%	33%	31%	25%	ID	33%	28%	32%	32%	32%
pa1204	36%	33%	32%	27%	33%	ID	37%	31%	30%	33%
pa0949	31%	31%	27%	28%	28%	37%	ID	30%	30%	33%
pa4975	36%	35%	36%	33%	32%	31%	30%	ID	56%	37%
pa1224	37%	33%	35%	28%	32%	30%	30%	56%	ID	39%
pa1225	37%	37%	37%	35%	32%	33%	33%	37%	39%	ID

One of the most commonly used approaches to study gene function is the characterization of the proteins encoded by these genes. This includes the manipulation and expression of gene sequences such as gene cloning, protein production and characterization.

The pET vector is an expression vehicle developed for the cloning and expression of recombinant proteins in *E. coli* (Dubendorf and Studier, 1991, Studier et al., 1990). This system, in particular the pET28b(+) vector (Figure 3.1), has been already used to successfully clone and express the azoreductase genes 1, 2 and 3 from *P. aeruginosa* PAO1 (Wang et al., 2007, Ryan et al., 2010a).

The characterization of specific gene products requires the production of the corresponding soluble proteins in a sufficient amount for biochemical and enzymatic analyses. This is often difficult and sometimes not possible to achieve directly in the natural hosts. Because of this, recombinant protein production in a different host organism is frequently the only applicable procedure. The host genetic background for recombinant protein production is extremely important. Expression strains are deficient in the most harmful natural proteases, maintain the expression plasmid stably and confer the genetic elements relevant to the expression system. To express heterologous proteins, different expression hosts have been developed (Baneyx and Mujacic, 2004). *E. coli*, and in particular the strain BL21, is one of the most used expression hosts due to its simplicity, its low cost and fast-high growth density, as well as the knowledge of its genomic background. Recombinant protein production is normally induced from a plasmid introduced into a host with a compatible background.

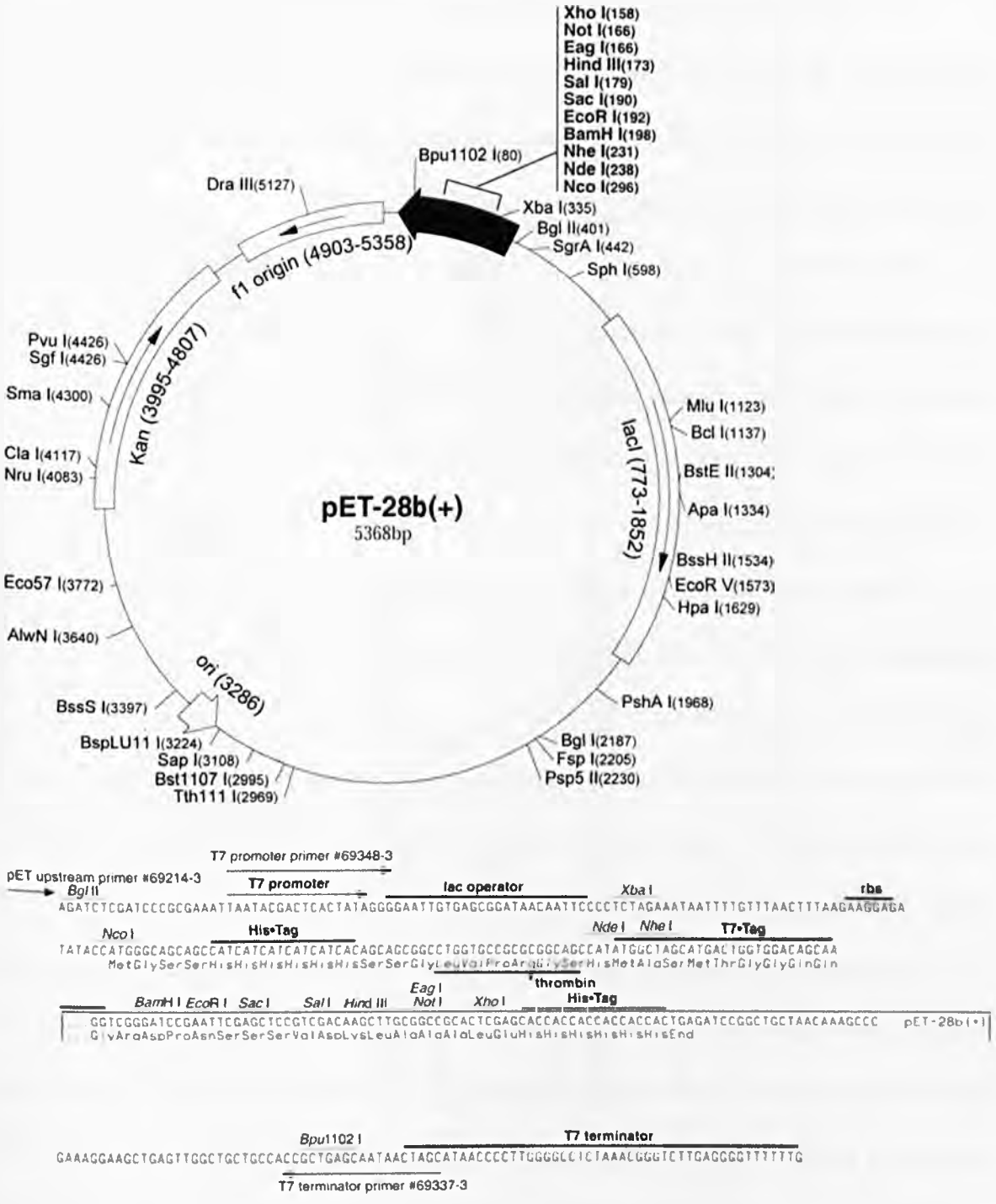


Figure 3.1 pET28b(+) vector map.

a) pET28b(+) vector map showing the multiple restriction sites (black arrow). NdeI and SacI restriction sites were chosen to insert the gene sequences. **b)** Multiple restriction site nucleotide sequence showing the position of each restriction site as well as the HisTag coding region (used to encode a hexahistidine residue to use in the following recombinant protein purification) and the thrombin cleavage site (used to remove the HisTag after the recombinant protein purification). Figure adapted from Novagen website.

Expression plasmids are usually characterized by the presence of genetic elements such as origin of replication (*ori*), antibiotic resistance markers, transcriptional promoter, translational initiation regions (TIRs) and transcriptional and translational terminators (Figure 3.2 **a**) (Sørensen and Mortensen, 2005).

The pET expression system includes hybrid promoters, multiple cloning sites for the incorporation of gene sequences, protease cleavage sites and a number of genetic background modifications for various expression purposes. Expression requires a host strain encoding the T7 RNA polymerase (bacteriophage T7 gene 1), under the control of the IPTG inducible *lacUV5* promoter.

Target genes are initially cloned into bacterial hosts that do not contain the T7 RNA polymerase gene (eg. *E. coli* JM109ES strain) avoiding plasmid instability due to the production of proteins potentially toxic to the host cell. To produce the target proteins, the plasmids are then transfected into an expression host containing a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter (eg. *E. coli* BL21 strain) (Sørensen and Mortensen, 2005).

Promoter induction can be either thermal or chemical and the most common inducer is the sugar molecules IPTG (Hannig and Makrides, 1998). Basal transcription, in the absence of inducer, is minimised by the presence of a suitable repressor. A copy of the *lacI* gene is present in the *E. coli* genome and in the plasmid sequence. Without IPTG, the lacI tetramer is synthetized from both host and plasmid genes. This leads to the repression of the *lacUV5* host promoter and the *T7/lac* hybrid promoter encoded by the expression plasmid. T7 RNA polymerase is transcribed when IPTG binds the *lac* operator and triggers the release of tetrameric LacI from the *lac* operator. Transcription of the target gene from the *T7/lac* hybrid promoter is subsequently initiated by T7 RNA polymerase (Figure 3.2 **b**) (Sørensen and Mortensen, 2005).

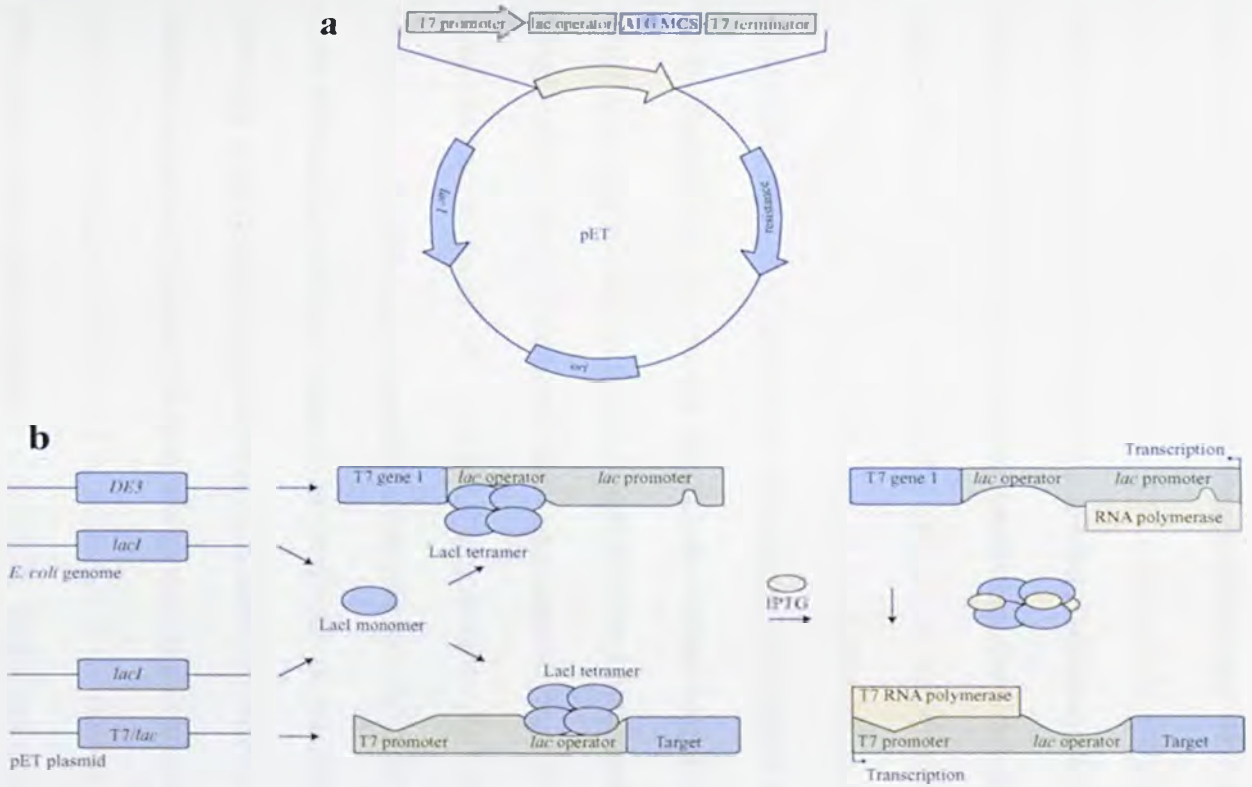


Figure 3.2 pET recombinat expression mechanism.
(**a**) pET expression vector showing the general plasmid configuration, which includes the genetic elements for gene cloning and expression. (**b**) Representation of the macromolecular situations prior to and after IPTG induction. The LacI molecules produced by the host and plasmid form tetramers and bind the *lac* operators preventing the target gene expression. Then IPTG binds and triggers the release of tetrameric LacI from the *lac* operator and transcription of the target gene is subsequently initiated by T7 RNA polymerase. Figure taken from Sorensen et al. (2005).

Advances in genomics, proteomics and bioinformatics, have led to an exponential increase of the number of proteins being produced using recombinant techniques and the rapid identification of proteins with a potential application as therapeutic, diagnostic or industrial enzymes (Korf et al., 2005).

The characterization of new proteins would not be feasible if specific purification procedures were not developed for each individual protein. One of the most applied methods for protein purification includes the use of affinity tags which enables different proteins to be purified (Arnau et al., 2006b).

Affinity tags consist of additional amino acid sequences with high affinity for specific biological or chemical ligand linked on a solid support. One approach is the purification of tagged fusion recombinant proteins based on the use of chelated metal ions as affinity ligands and which are complexed with immobilized chelating agents (Immobilized Metal-Ion Affinity Chromatography, IMAC). The most commonly used transition metal ions are Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Fe^{3+} (Porath et al., 1975). Amongst all the amino acids that can participate in the binding process (including glutamic acid, aspartic acid, arginine, lysine, tyrosine, histidine, cysteine and methionine), the most common used is a tag of histidine residues to promote protein binding by IMAC (Arnold, 1991, Sulkowski, 1989).

For structural studies, more than 60% of proteins produced incorporate polyhistidine tags, making them the most widely used affinity tags (Derewenda, 2004). The His-Tags can be placed at either N- and C-terminus or in a region with appropriate surface exposure to allow binding (Müller et al., 2002). This enables the purification of the target protein from the crude extract of the host cells in a single step (Porath, 1992, Abdullah and Chase, 2005). The binding specificity of this system enables the purification of proteins under both native and denaturating conditions (Arnau et al., 2006a).

The most common form of HisTag consists of six consecutive histidine residues,

which provide multiple metal-binding sites and the most common IMAC support is nitrilotriacetic acid (NTA) as a ligand for immobilizing metals such as nickel in affinity chromatography (Ni–NTA). The hexahistidine tag of the recombinant protein interacts with nickel, which strongly binds and immobilizes the protein within the matrix (Figure 4.2). In this way only proteins with the HisTag are immobilized and hold in the column, whereas all other proteins pass through the matrix, although non-specific binding may be a practical problem.

The binding between His-tagged flavoproteins and Ni-NTA matrix is represented by a colour change of the matrix, which goes from light blue (free form) to yellow (bound form). In the same way, when the protein is released from the matrix, its colour reverts to the original light blue. In this way the processes of immobilization and elution of tagged azoreductase proteins, which have been shown to be flavoproteins, can be visually monitored.

To complete the recombinant protein purification, the removal of the tag can be sometimes required. To do this, a linker region, containing a sequence specific for protease cleavage (e.g. thrombin cleavage site), is usually present between the tag and the native protein sequence (Figure 3.3). In this way the tag can be removed, leaving only a few extra amino acid residues (GSH) at the N-terminus of the protein sequence (Figure 3.4).

Previous studies have described the production and purification of three azoreductases (paAzoR1-3) from *P. aeruginosa* PAO1 using the pET system and Ni-NTA methods described above (Wang et al., 2007, Ryan et al., 2010a). This chapter describes the cloning of the five putative *P. aeruginosa* PAO1 azoreductase genes (*pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*) and the production, purification and molecular weight determination of the four putative azoreductase proteins PA0949, PA1204, PA2280 and PA2580 from *P. aeruginosa* PAO1.

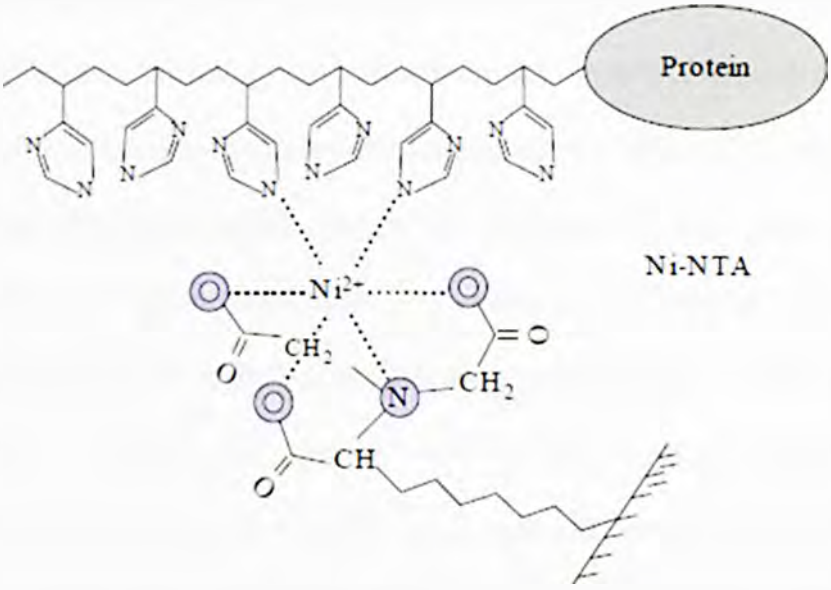


Figure 3.3 Model of the interaction between residues in the His tag and the metal ion in the NiNTA IMAC

Figure showing the interaction, and consequently the binding, between the nitrogen atoms (N) of histidine residues present on the protein, and nickel ions immobilized on matrix. Figure taken from Block et al (Block et al., 2009).

```
                    10         20         30         40         50
                    ....|....| ....|....| ....|....| ....|....| ....|....|
HisTag-PA0949       MGSSHHHHHH SSGLVPRGSH MSSPYILVLY YSRHGATAEM ARQIARGVEQ
                    60         70         80         90         100
                    ....|....| ....|....| ....|....| ....|....| ....|....|
HisTag-PA0949       GGFEARVRTV PAVSTECEAV APDIPAEGAL YATLEDLKNC AGLALGSPTR
                    110        120        130        140        150
                    ....|....| ....|....| ....|....| ....|....| ....|....|
HisTag-PA0949       FGNMASPLKY FLDGTSSLWL TGSLVGKPAA VFTSTASLHG GQETTQLSML
                    160        170        180        190        200
                    ....|....| ....|....| ....|....| ....|....| ....|....|
Histag-PA0949       LPLLHHGMLV LGIPYSEPAL LETRGGGTPY GASHFAGADG KRSLDEHELT
                    210        220
                    ....|....|....|....|
HisTag-PA0949       LCRALGKRLAETAGKLGS
```

Figure 3.4 His tagged PA0949 protein sequence.

Amino acid sequence of the His-tagged PA0949 protein showing the protein amino acid composition (black amino acids at position 21-218), the hexahistidine tag amino acids (red amino acids at position 4-10) and the thrombin cleavage site (blue amino acids at position 14-19). After thombin cleavage of the HisTag, only a few extra amino acid residues (GSH) are present at the N-terminus of the protein sequence. The resulting protein shows a molecular weight of 22.93 KDa.

3.2. Results

3.2.1. Sequencing of putative *P. aeruginosa* PAO1 azoreductase genes

The five putative *P. aeruginosa* PAO1 azoreductase genes *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975* were amplified and sequenced. For each gene a single PCR product of the correct size was obtained (Figure 3.5 and Table 3.3). All PCR products were then purified and sequenced. The forward and reverse sequences obtained for each gene were aligned and compared with the corresponding gene sequences available in the Pseudomonas Genome Database (Winsor et al., 2011), showing the correspondence between genes sequenced and database data (alignment data shown in APPENDIX I).

3.2.2. Cloning of putative *P. aeruginosa* PAO1 azoreductase genes

3.2.2.1. PCR amplification, ligation and transformation

The five putative azoreductase genes *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975* were amplified from *P. aeruginosa* PAO1 total genomic DNA. For the gene *pa0949* the start codon was changed from TTG to ATG by inserting the appropriate nucleotide in the forward primer sequence in order to include NdeI restriction site before the start codon of this gene. The PCR results show that a single PCR product of the expected size was obtained for each gene amplified (Figure 3.6 and Table 3.4). All PCR products were then purified and quantified for the following restriction enzymes digestion.

The Purified PCR products and pET28b(+) plasmid, extracted from *E. coli* JM109ES cells, were quantified and digested with NdeI and SacI restriction enzymes. The plasmids were then analysed on 1% w/v agarose (Figure 3.7) showing the linearization of the digested plasmids (defined single band), compared with the undigested plasmid, which shows bands typical of undigested plasmid forms (supercoiled, open-circular and linear). The difference between the digested and undigested samples indicates the complete plasmid digestion obtained with the two restriction enzymes.

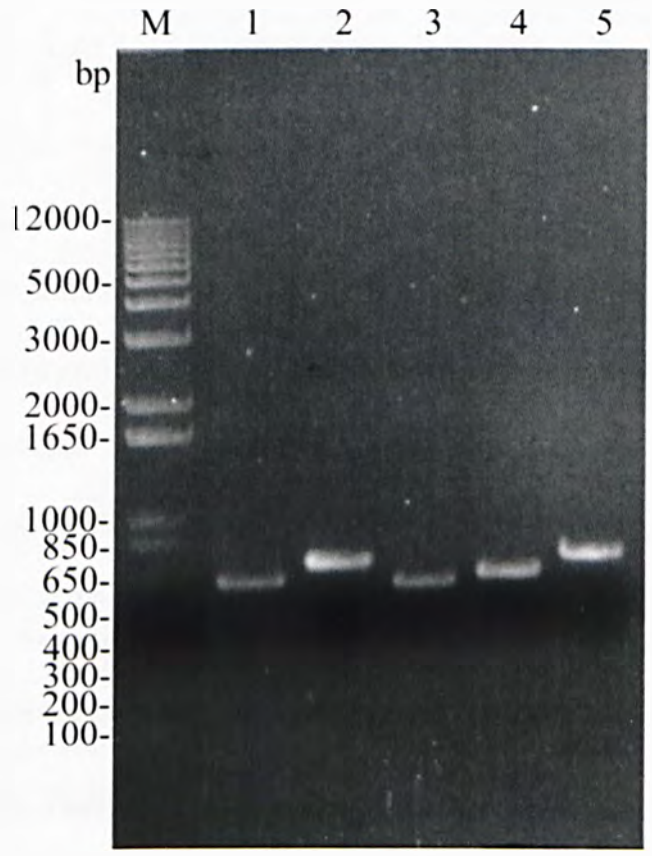


Figure 3.5 *P. aeruginosa* PAO1 putative azoreductase PCR products for sequencing. Picture showing the 1% w/v agarose gel of the putative azoreductase genes amplified from the total *P. aeruginosa* PAO1 genomic DNA using HF Taq polymerase, as described in section 2.4.1. Samples shown are 1 Kb Plus DNA Ladder (lane M) *pa2580* (lane 1) *pa2280* (lane 2), *pa1204* (lane 3), *pa0949* (lane 4) and *pa4975* (lane 5) genes. The PCR products obtained for all genes amplified were of the expected size (Table 3.3).

Table 3.3 Interpolated size of putative azoreductase genes amplified for sequencing. PCR product sizes calculated based on the bands migration on 1% w/v agarose gel as described in section 2.3.3. The interpolated sequencing PCR product lengths correspond to the expected lengths (Table 2.3) with an error between 0% and 3.6%.

PCR product	Measured length of sequencing PCR products (bp)
pa0949	675
pa1204	650
pa2280	780
pa2580	665
pa4975	796

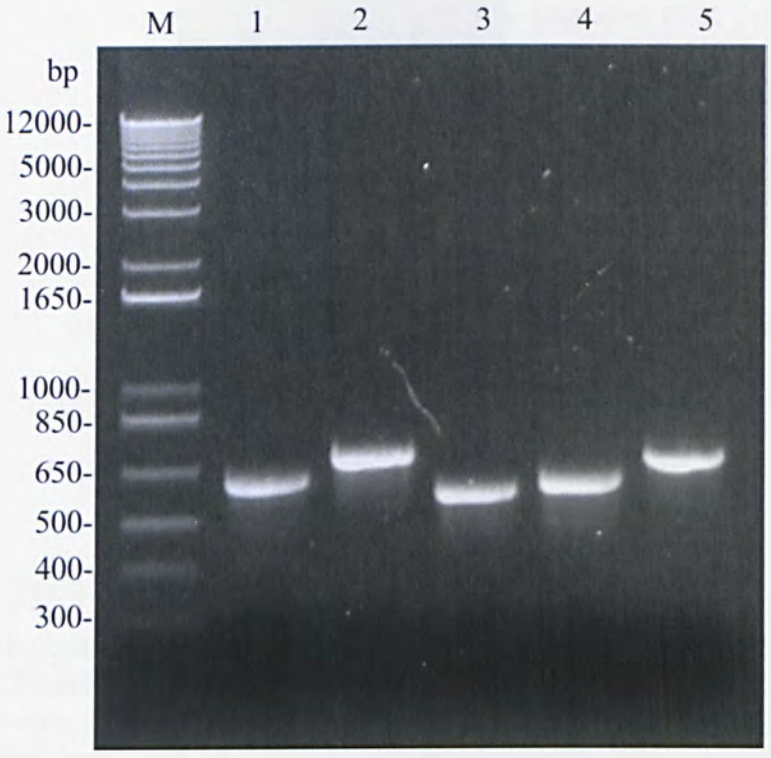


Figure 3.6 *P. aeruginosa* PAO1 putative azoreductase PCR products for cloning.
Picture showing the 1% w/v agarose gel of the putative azoreductase amplified from the total *P. aeruginosa* PAO1 genomic DNA using HF taq polymerase. Samples shown are 1 Kb Plus DNA Ladder (lane M), *pa2580* (lane 1) *pa2280* (lane 2), *pa1204* (lane 3), *pa0949* (lane 4) and *pa4975* (lane 5) genes. The PCR products obtained for all genes amplified were of the expected size (Table 3.3).

Table 3.4 Interpolated size of putative azoreductase genes amplified for the cloning process.
PCR product sizes calculated based on the bands migration on 1% w/v agarose gel as described in section 2.3.3. The interpolated cloning PCR product lengths correspond to those in the database (Table 2.7) with an error between 0.18% (*pa2580*) and 3% (*pa2280*).

PCR product	Measured length of cloning PCR products (bp)
pa0949	590
pa1204	565
pa2280	672
pa2580	590
pa4975	702

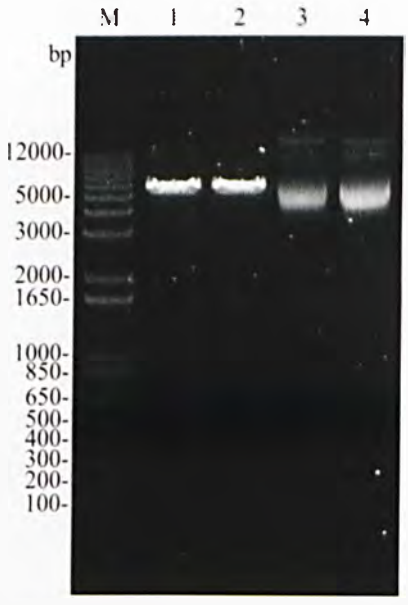


Figure 3.7 Plasmid restriction endonuclease digestions
1% w/v agarose gels showing the pET28b(+) plasmid digestion products using NdeI and SacI restriction enzymes. Digested samples (lanes 1 and 2) shown a linearized plasmid DNA comparing with undigested samples (lanes 3 and 4), which shown the undigested plasmid forms (supercoiled, open-circular and linear). 1kb plus DNA ladder (lane M) was used as standard for both analyses.

To prevent plasmid self-ligation, these were then treated with a phosphatase enzyme to remove the phosphate group at the 5' end. Digested PCR products were not treated with phosphatase, leaving the 5' phosphate group necessary for the ligation process. The phosphatase treated samples were purified and quantified once more in preparation for the ligation process.

PCR products and plasmids were ligated together obtaining the five constructs, one for each gene of interest (eg. pET28b(+)-pa0949). The ligation reaction was directly used to transform E. coli JM109ES chemically competent cells.

3.2.2.2. Screening and sequencing of clones

Colonies of positive clones as well as colonies of cell transfected with empty plasmids, were picked and grown in LB broth containing kanamycin. A few colonies for each gene and one for the empty plasmid were screened via PCR. The PCR products were analysed on 1% w/v agarose gel and results showed a single PCR product of the expected size for all colonies analysed. No amplification products were obtained for the empty plasmids.

Figure 3.8 shows the amplification products obtained for the clones for the genes *pa0949* (a) and *pa1204* (b) and the absence of the insert in the empty plasmid (lane 7 in each gel). Equivalent results were obtained for all other genes cloned (*pa2280*, *pa2580* and *pa4975*) indicating the success of the cloning process for all of the genes of interest (data shown in APPENDIX II).

The size (bp) of each PCR product was determined based on their migration compared with the migration of the standard bands on agarose gel. Determination of PCR product size is shown only for pa0949 and pa1204 genes (Figure 3.9) and similar graphs have been obtained also for pa2580, pa2280 and pa4975.

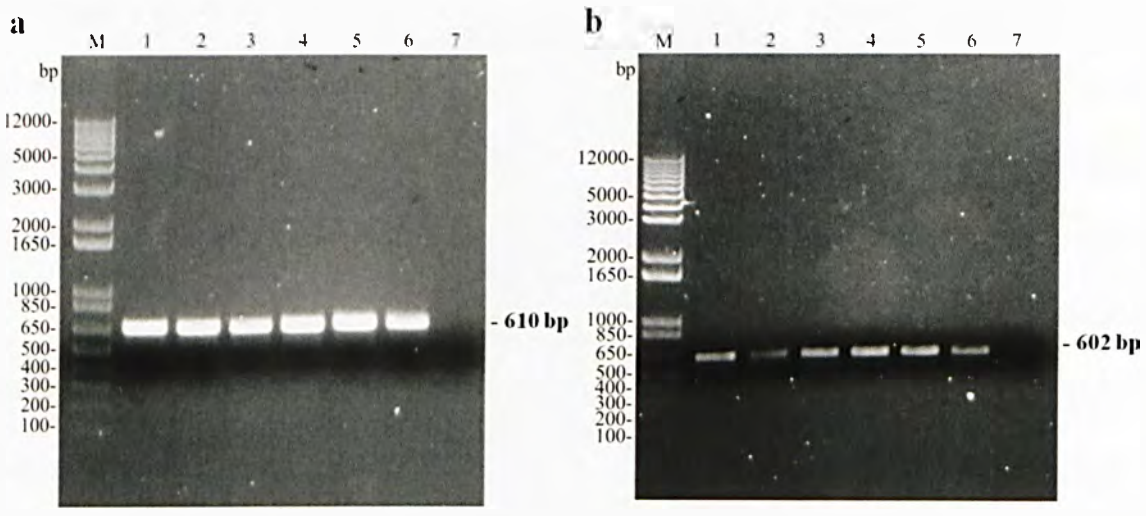


Figure 3.8 *E. coli* JM109ES clones screening.
1% w/v agarose gels showing the PCR products for the gene *pa0949* (**a**) and *pa1204* (**b**) obtained from six *E. coli* JM109ES colonies for the transfected samples (line 1 to 6) and one for the empty plasmid used as transformation control (line 7). The PCR was performed using directly the *E coli* JM109ES culture previously transfected with pET28b(+) plasmids and containing the cloned genes. 1kb plus DNA ladder (line M) was used as standard.

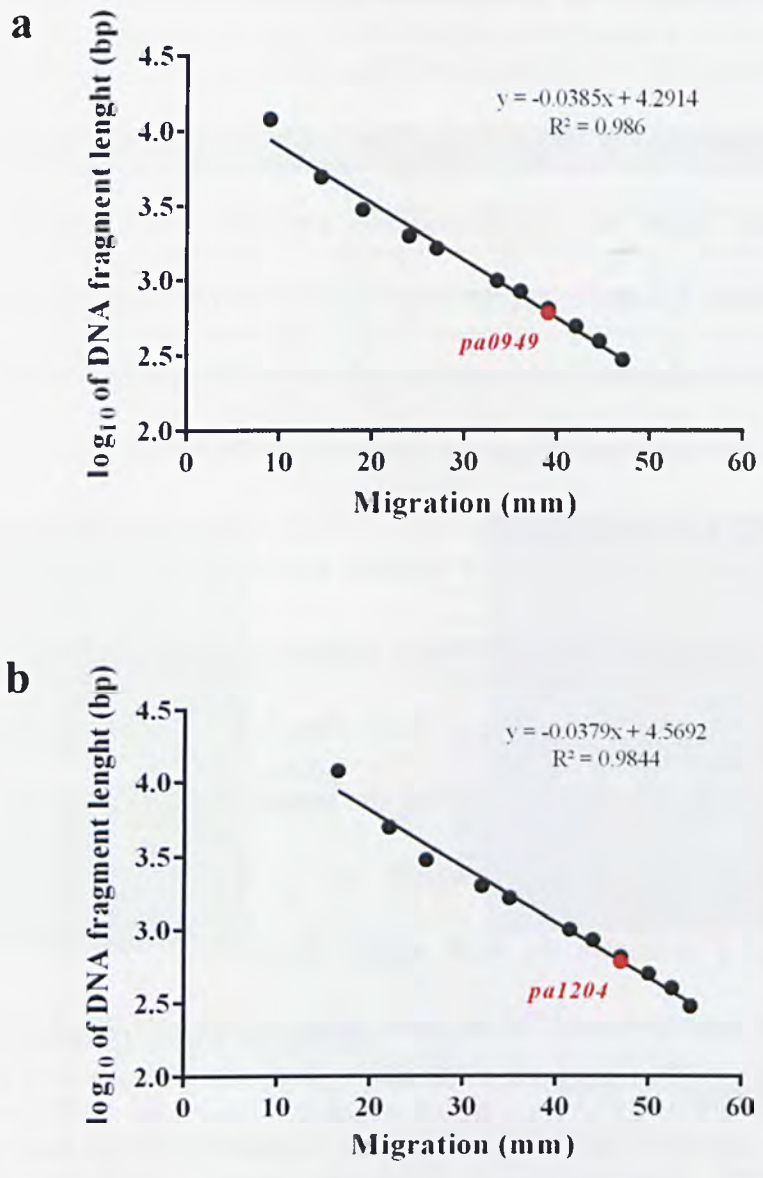


Figure 3.9 Determination of PCR product sizes
Graphs showing the $\log_{10}$ of PCR product size for the gene *pa0949* (**a**) and *pa1204* (**b**). Log_{10} of the standard size of the bands was calculated and associated to the migration (mm) of each band (black circles). Linear regression for all bands for the standard were calculated and the $\log_{10}$ of the PCR products sizes were interpolate based on their migration (red circles). Graphs generated with GraphPad Prism 6.

The PCR products obtained for all gene cloned shown to be of the expected size in comparison with those given by Pseudomonas Genome Database with an error between 0.67 and 7.3% (Table 3.5).

The plasmids of the PCR confirmed clones were extracted and sequenced. Sequencing results show the correspondence between genes cloned and those in Pseudomonas Genome Database, confirming the success of the cloning process for all five genes. The sequence analyses also showed that no PCR mistakes occurred during the amplification process (sequence alignments shown in APPENDIX III).

3.2.2.3. *E. coli* BL21 transformation and colony screening

pET28b(+) plasmids extracted from positive cloned (confirmed via PCR and sequencing) were transfected into *E. coli* BL21 chemically competent cells. Colony PCR was performed on five BL21 clones showing the presence of insert for the positive clones and no insert for the negative (empty plasmid).

The Figure 3.10 shows the PCR results for genes *pa0949* (**a**) and *pa1204* (**b**). Equivalent results were obtained for all other genes cloned *pa2280*, *pa2580* and *pa4975* (data shown in APPENDIX IV).

3.2.3. Small scale production of putative *P. aeruginosa* PAO1 recombinant azoreductase proteins

Transfected *E. coli* BL21 cells with pET28b(+) plasmids each containing one of the four putative azoreductase genes *pa1204*, *pa2580*, *pa2280* and *pa0949* were used to produce the corresponding recombinant proteins PA1204, PA2580, PA2280 and PA0949 at small scale (50 ml). These were compared with a negative control (empty plasmid), which was always processed in the same way of the expression samples.

Table 3.5 Interpolated size of putative azoreductase genes cloned.
PCR product sizes calculated based on the bands migration on 1% w/v agarose gel and compared with the predicted gene lengths annotated in Pseudomonas Genome Database (Winsor et al., 2011). The interpolated PCR lengths corresponded to those in the database with and error between 0.67% (*pa2580*) and 7.3% (*pa1204*).

PCR product	Measured length (bp)	Predicted length (bp)
pa0949	610	597
pa1204	602	558
pa2280	700	693
pa2580	595	591
pa4975	682	705

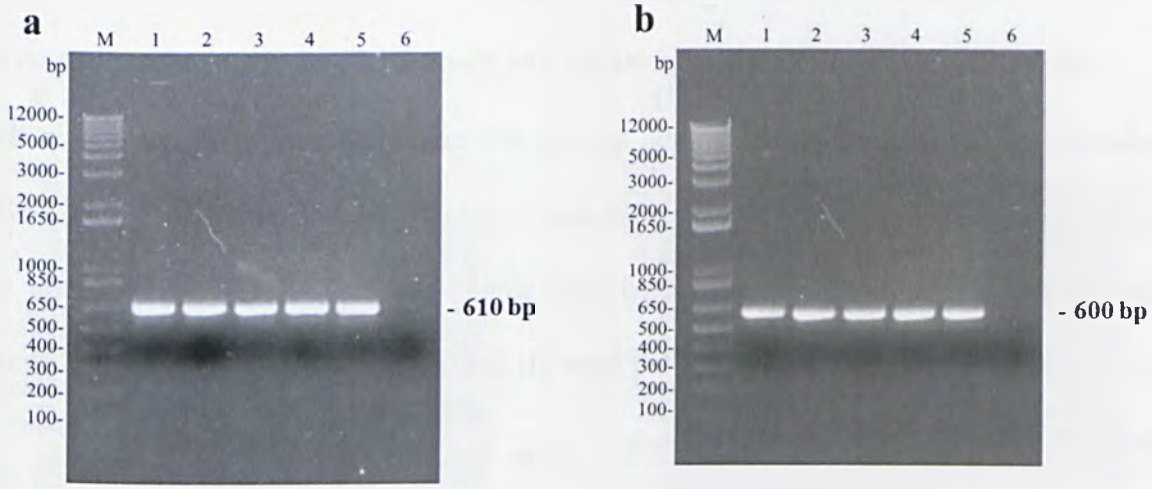


Figure 3.10 *E. coli* BL21 clones screening.
1% w/v agarose gels showing the PCR products for the gene *pa0949* (**a**) and *pa1204* (**b**) obtained from six BL21 colonies for the transfected samples (lane 1 to 5) and one for the empty plasmid used as transformation control (lane 6). The PCR was performed using directly the *E. coli* BL21 culture previously transfected with pET28b(+) plasmids containing cloned genes. 1 kb plus DNA ladder (lane M) was used as standard.

The small scale protein production results show the overexpression of the genes *pa1204, pa2580, pa2280* and *pa0949* and consequently the over production of the corresponding proteins (Figure 3.11). This was clearly evident comparing the clones (Figure 3.11, lanes 4-6) with the negative control (Figure 3.11, lanes 1-3).

The clones BL21-*pa1204*, -*pa2580* and -*pa2280* showed evidence of protein over production represented by a single and intense band of the correct molecular weight (22, 24 and 28 KDa respectively), which was not present in the negative controls.

In contrast to the other three proteins, the clone BL21-*pa0949* showed the over production of two distinct polypeptides, also in this case in this case not present in the negative control, represented by two intense and distinct bands of approximately 24 and 19 KDa. As these results were not expected (one single band, representing one protein of approximately 24 KDa was expected) two more BL21-*pa0949* clones were tested and same results were obtained (data not showed).

Comparison of the lysate fractions show that PA1204 and PA2580 proteins were predominantly soluble (Figure 3.11 **a** and **b**), whereas PA0949 was equally present in the soluble and insoluble fractions (Figure 3.11 **d**). The protein PA2280, differently from the three previously described, was predominantly insoluble (Figure 3.11 **c**). However, recombinant PA2280 was present in the soluble fraction, suggesting that a large scale production would be worthwhile. Based on these results, the process was scaled up to 500 ml culture for all four proteins.

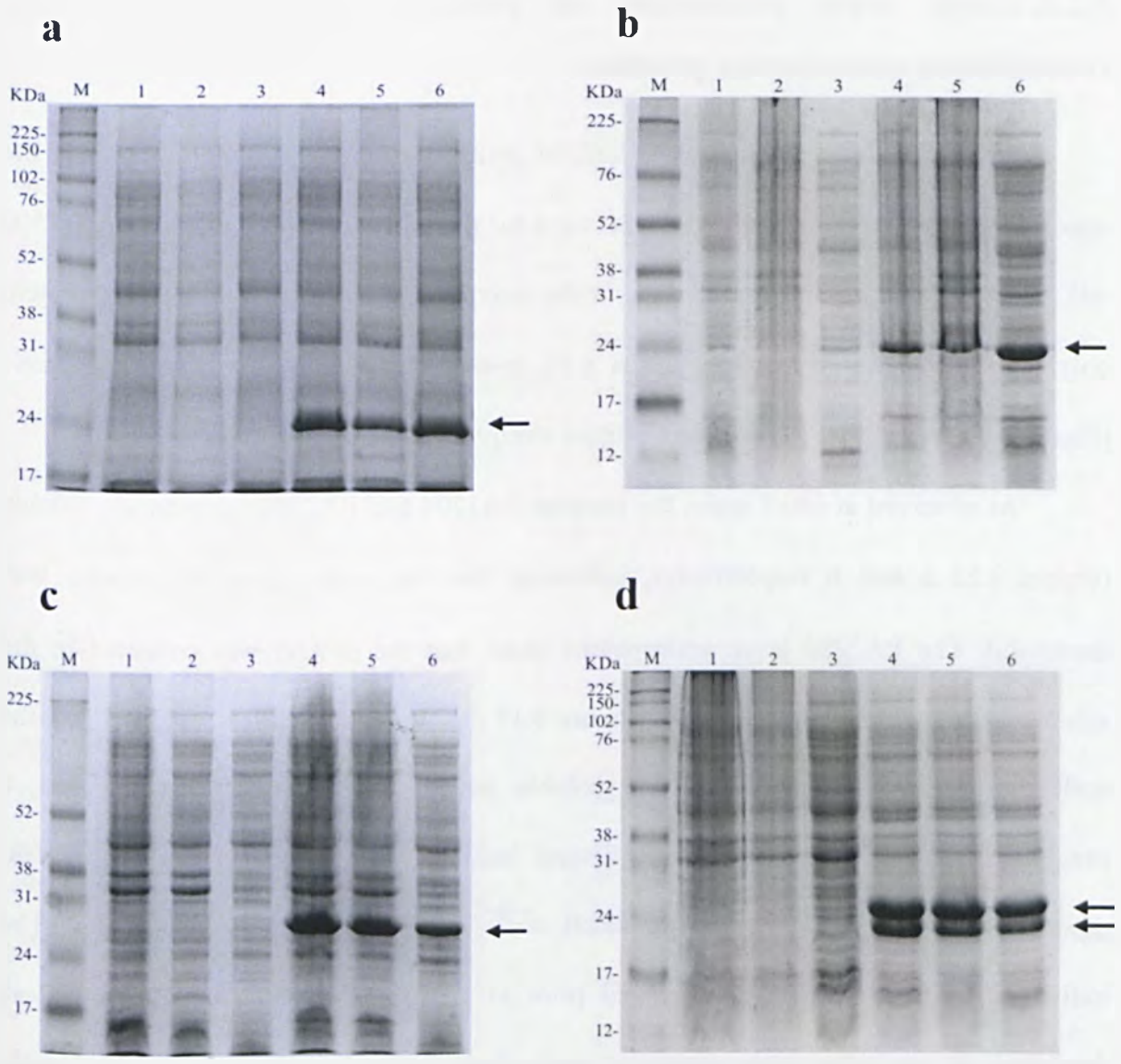


Figure 3.11 Small scale production of putative *P. aeruginosa* PAO1 recombinant azoreductase proteins.

SDS-PAGE showing the PA1204 (**a**), PA2580 (**b**), PA2280 (**c**) and PA0949 (**d**) protein production at small scale (50 ml culture) using *E. coli* BL21 tranformed with pET28b(+) plasmid containing *pa1204, pa2580, pa2280* and *pa0949* genes respectively. Protein production of azoreductase clones (lanes 4-6) was compared with the negative control (*E. coli* BL21 containing empty plasmid, lanes 1-3), showing an over production of the corresponding protein only for the azoreductase clones (black arrows). One intense band was obtained for PA1204 (22 KDa), PA2580 (24 KDa), PA2280 (28 KDa) and two bands for PA0949 (19 and 24 KDa) sample. In all cases, the recombinant protein produced was collected part in the soluble (lanes 6) and part in the insoluble fraction (lanes 5). Samples shown are Amersham ECL Full-Range Rainbow Molecular Weight Marker (lane M), negative control whole cell lysate (lane 1), negative control insoluble fraction (lane 2), negative control soluble fraction (lane 3), clones whole cell lysate (lane 4), clones insoluble fraction (lane5) and clones soluble fraction (lane 6). Ten µl of sample were loaded in each well.

3.2.4. Large scale production of putative *P. aeruginosa* PAO1 recombinant azoreductase proteins

The same clones for the genes *pa1204*, *pa2580*, *pa2280* and *pa0949* used for the small scale protein productions were also used for the large scale protein production (500 ml). The results of all four proteins show the overproduction of the recombinant protein only for the clones (Figure 3.12, lanes 3-5), in comparison with the negative controls (Figure 3.12, lanes 1-3), for which no protein overproduction was observed.

As observed at small scale, the proteins PA1204 and PA2580 were mostly soluble (Figure 3.12 **a** and **b** respectively), indicating that the scale up of the process was successful. The PA2280 large scale results show that the protein was collected in the soluble fraction, remaining insoluble (Figure 3.12 **c**). This indicates that, for PA2280, the scale-up had not been successful for soluble protein generation. For this reason, a pragmatic approach was taken where several individual 100 ml cultures were used to produce this protein following the small scale protein production procedures. The individual 100 ml cultures were mixed prior to centrifugation and then processed as described for the large scale, obtaining equivalent results to those of the small scale production described in the previous section. The results obtained for PA0949 were equivalent to those obtained on a small scale, showing two distinct strong bands in both soluble and insoluble fraction at similar level (Figure 3.12 **d**).

Comparison of soluble and insoluble fractions (Table 3.6) indicates that sufficient soluble PA1204, PA2580, and PA0949 were produced at large scale, whereas combination of multiple small scale cultures was necessary to produce soluble PA2280 protein for further analysis.

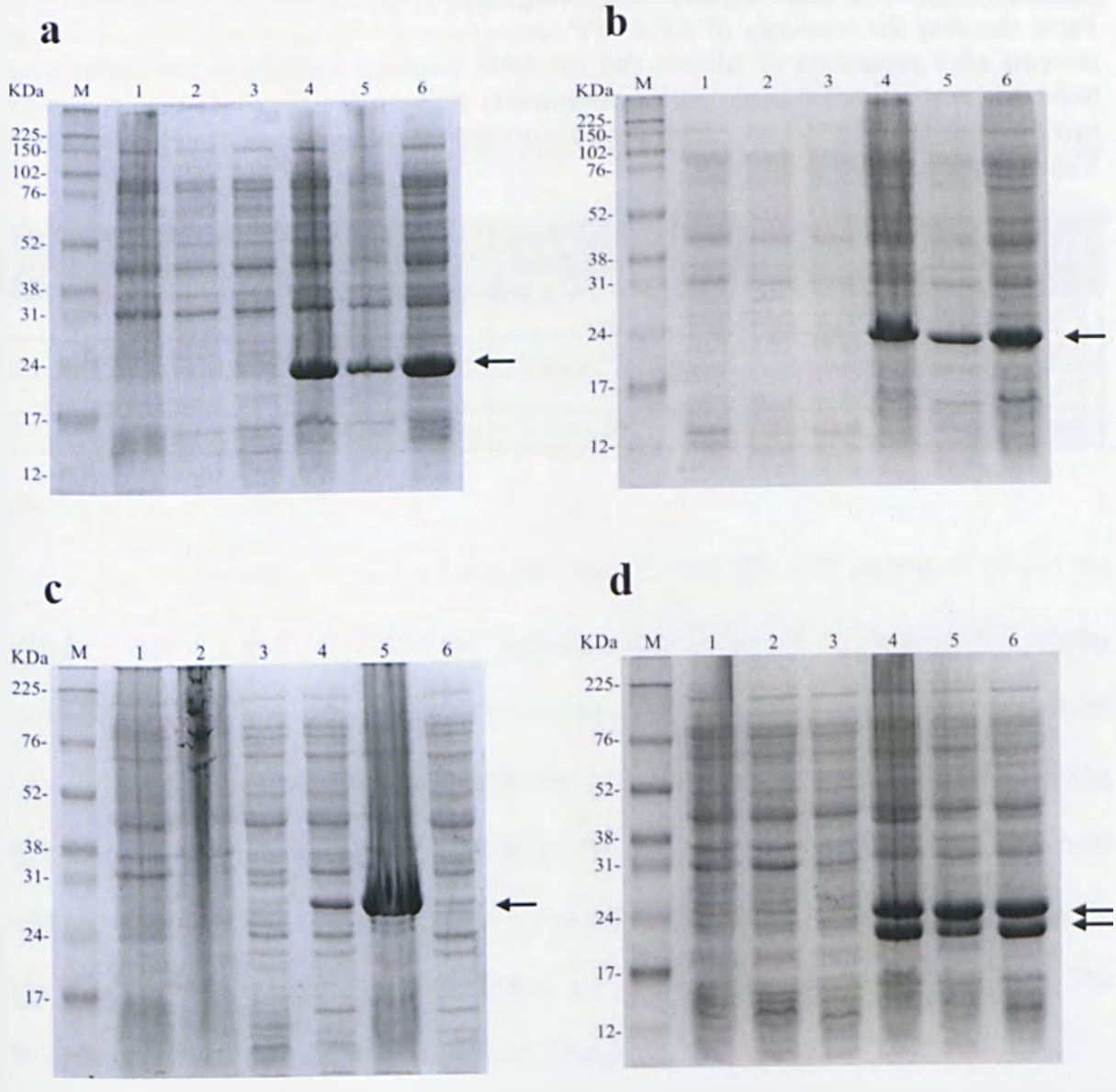


Figure 3.12 Large scale production of putative *P. aeruginosa* PAO1 recombinant azoreductase proteins.

SDS-PAGE showing the PA1204 (**a**), PA2580 (**b**), PA2280 (**c**) and PA0949 (**d**) protein production at large scale (500 ml culture) using *E. coli* BL21 tranformed with pET28b(+) plasmid containing *pa1204, pa2580, pa2280* and *pa0949* genes respectively. Protein production of azoreductase clones (lanes 4-6) was compared with the negative control (*E. coli* BL21 containing empty plasmid, lanes 1-3), showing an over production of the corresponding protein only for the azoreductase clones (black arrows). One intense band was obtained for PA1204 (22 KDa), PA2580 (24 KDa), PA2280 (28 KDa) and two bands for PA0949 (19 and 24 KDa) sample. The recombinant protein PA1204, PA2580 and PA0949 were collected part in the soluble (lanes 6) and part in the insoluble fraction (lanes 5), whereas PA2280 was collected completely in the insoluble fraction (gel **c**, lane 5). Samples shown are Amersham ECL Full-Range Rainbow Molecular Weight Marker (lane M), negative control whole cell lysate (lane 1), negative control insoluble fraction (lane 2), negative control soluble fraction (lane 3), clones whole cell lysate (lane 4), clones insoluble fraction (lane5) and clones soluble fraction (lane 6). Ten µl of sample were loaded in each well.

Table 3.6 Solubility index of putative *P. aeruginosa* PAO1 azoreductase proteins.
Table showing the solubility of putative *P. aeruginosa* PAO1 azoreductase recombinant proteins after separation of soluble and insoluble fractions from the whole cell lysate. Solubility was estimated based on the comparison between protein bands obtained in the two fractions at small (50 ml culture) and large (500 ml culture) scale protein production. Values expressed in percent (%).

Protein	Small scale		Large scale	
	Soluble fraction	Insoluble fraction	Soluble fraction	Insoluble fraction
PA1204	60	40	70	30
PA2580	70	30	70	30
PA2280	40	60	0	100
PA0949	50	50	50	50

3.2.5. Purification of putative *P. aeruginosa* PAO1 recombinant azoreductase proteins

The soluble fraction of the recombinant proteins PA1204, PA2580, PA2280 and PA0949 were loaded into a column containing the Ni-NTA matrix, which quickly changed colour from light blue to bright yellow, indicating the binding of the protein to the nickel beads. The reversion of the colour to the original state (indicating the release of the protein from the Ni-NTA matrix) was observed while increasing imidazole concentrations were used to elute completely the protein.

For the protein PA1204, after a first elution with 250 mM imidazole (W_{250}) the nickel colour was still bright yellow, indicating a large amount of protein still present within the matrix. For this reason, 500 mM imidazole was used for two more elutions (W_{500} a and b). Each elution was then analysed on SDS-PAGE (Figure 3.13 **a**) showing that most of the protein was collected in the W_{250} and in the first W_{500} elutate. A small amount of protein was also present in the W_{50} and W_{100}, however, the protein collected in the W_{500} and W_{250} showed a higher degree of purity compared with the W_{50} and W_{100}. The W_{500} (first and second) and W_{250} were therefore combined and used for further analysis.

Also in the case of the PA2580 protein, after a first elution with 250 mM imidazole (W_{250}) the nickel colour remained yellow. One more elution using 250 mM imidazole (W_{250} b) was performed and the NTA resin was restored to its blue colour indicating complete elution of the protein. The analysis of each elutate on SDS-PAGE (Figure 3.13 **b**) showed that the protein started to be eluted with 50 mM imidazole, increasing gradually with 100 and 250 mM imidazole. Some protein was also present in the W_{50}, however most of the protein was eluted with 100 and 250 mM imidazole, which were combined and used for further analysis.

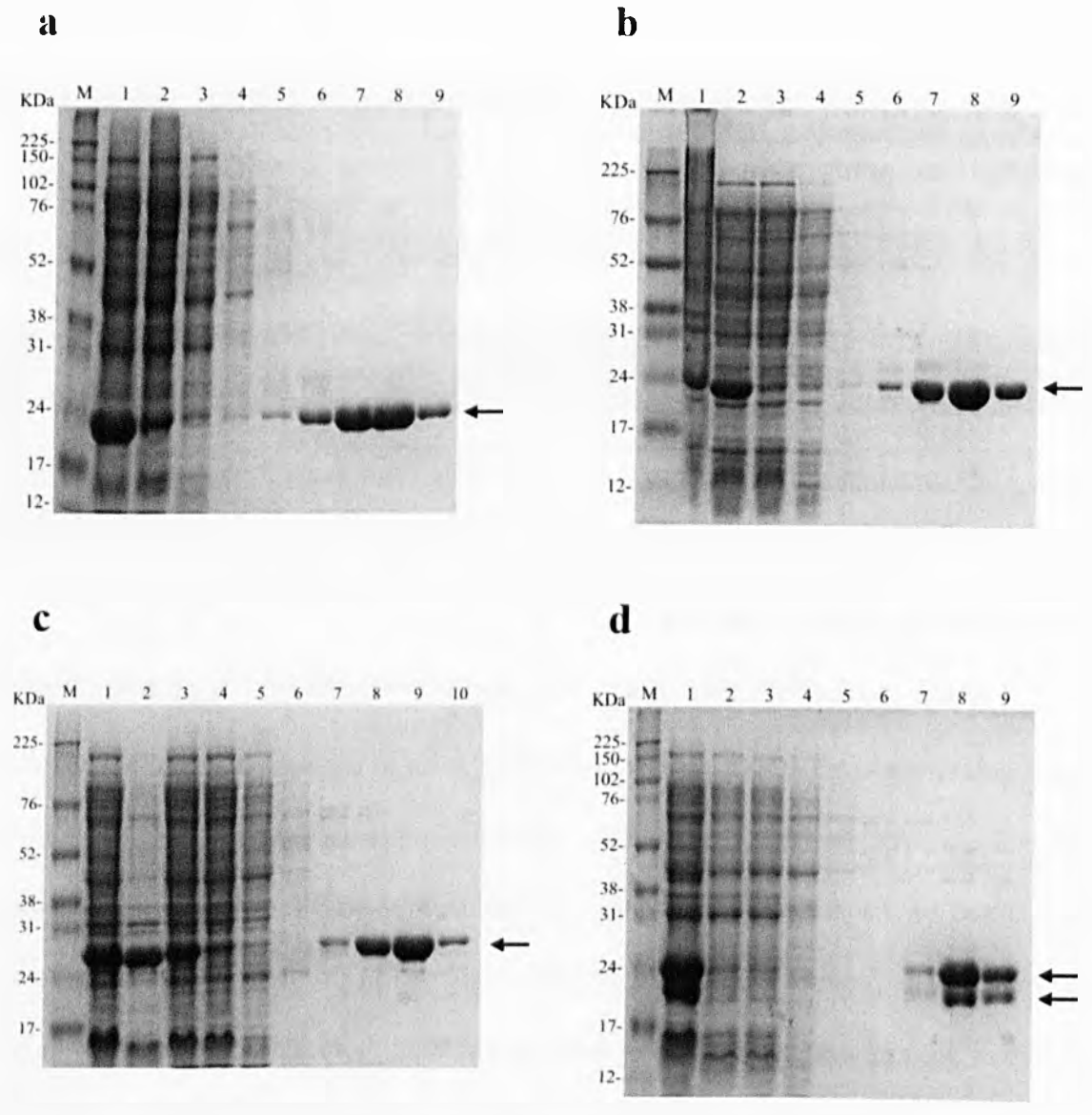


Figure 3.13 Purification of putative *P. aeruginosa* PAO1 recombinant azoreductase protein using immobilised metal-ion affinity chromatography.

SDS-PAGE showing the Ni-NTA purification using soluble fractions of the putative *P. aeruginosa* PAO1 recombinant azoreductase protein lysates. The soluble fractions were loaded into the columns containing Ni-NTA and elution of the proteins (black arrows) was performed using standard buffer (20 mM TrisCl-HCl, 100 mM NaCl, pH 8) containing increasing imidazole concentrations (10, 25, 50, 100, 250 and 500 mM). (**a**) PA1204 protein (22 KDa) eluted 100, 250 and 500 mM imidazole (lane 6, 7, 8 and 9). Samples shown are Amersham ECL High-Range Rainbow Molecular Weight Marker (lane M), soluble fraction (lane 1), flow through (lane 2), W_{10} (lane 3), W_{25} (lane 4), W_{50} (lane 5), W_{100} (lane 6), W_{250} a (lane 7), W_{500} a (lane 8) W_{500} b (lane 9). (**b**) PA2580 protein (24 KDa) eluted with 50, 100 and 250 mM imidazole (lane 6, 7, 8 and 9). Samples shown are Amersham ECL High-Range Rainbow Molecular Weight Marker (lane M), insoluble fraction (lane 1), soluble fraction (lane 2), flow through (lane 3), W_{10} (lane 4), W_{25} (lane 5), W_{50} (lane 6), W_{100} (lane 7), W_{250} a (lane 8), W_{250} b (lane 9). (**c**) PA2280 protein (28 KDa) eluted with 50, 100 and 250 mM imidazole (lane 7, 8, 9 and 10). Samples shown are Amersham ECL High-Range Rainbow Molecular Weight Marker (lane M), whole cell lysate (lane 1) insoluble fraction (lane 2), soluble fraction (lane 3), flow through (lane 4), W_{10} (lane 5), W_{25} (lane 6), W_{50} (lane 7), W_{100} (lane 8), W_{250} a (lane 9), W_{250} b (lane 10). (**d**) PA0949 protein (19 and 24 KDa) eluted with 250 mM imidazole (lane 7, 8 and 9). Samples shown are Amersham ECL Full-Range Rainbow Molecular Weight Marker (lane M), soluble fraction (lane 1), flow through (lane 2), W_{10} (lane 3), W_{25} (lane 4), W_{50} (lane

5), W_{100} (lane 6), W_{250} a (lane 7), W_{250} b (lane 8) and W_{250} c (lane 9). Ten µl of sample were loaded in each well.

For the protein PA2280 two elutions using 250 mM imidazole (W_{250} a and b) were required to restore the nickel colour to light blue. A sample of each elution was analysed on SDS-PAGE (figure 3.13 **c**) showing that most of the protein was eluted with the first 250 mM imidazole elution (W_{250} a), however a considerable amount of protein was also eluted with 100 mM (W_{100} a) and minimal amount with 50 mM (W_{50}) and the second 250 mM elution (W_{250} b). The elutions W_{100} and W_{250} were combined and used for further analysis.

The PA0949 protein was completely eluted after three whashes with 250 mM imidazole (W_{250} a, b and c) as the nickel colour was restored to the original light blue. The SDS-PAGE (Figure 3.13 **d**) shown that no protein was eluted at concentrations of imidazole below 250 mM and most of it was eluted with the later 250 mM elution, which was used for further analysis. The SDS-PAGEs of the proteins PA1204, PA2580 and PA2280 shown a single band (22, 24 and 28 KDa respectively), whereas two distinct bands (24 and 19 KDa) were observed for PA0949. This confirms what was showed by the previous SDS-PAGE and indicates that both polypeptides of PA0949 still have the hexahistidine tag required for the affinity purification.

3.2.6. Buffer exchange dialysis and protein stocks preparation

Each of the recombinant proteins had been purified using elution buffer (20 mM Tris, 100 mM NaCl) containing high concentrations of imidazole. To reduce the imidazole concentration to less than 1 mM, buffer exchange dialysis was performed on the solutions containing the purified proteins, which were chosen based on the protein concentration and purity (table 3.7).

Table 3.7 Purified putative *P. aeruginosa* PAO1 azoreductase protein elutates used for the buffer exchange dialysis.

The table shows the eluates used for the buffer exchange dialysis and the imidazole concentration in each of them after the dialysis process.

Protein	Eluates used for dialysis	Imidazole final concentration
PA0949	W_{250}	0.3 mM
PA1204	W_{250} and W_{500}	0.3-0.6 mM
PA2280	W_{100} and W_{250}	0.1-0.3 mM
PA2580	W_{100} and W_{250}	0.1-0.3 mM

The dialysis process was performed using molecular porous membrane tubes containing the selected protein solutions, plunged into a large volume of dialysis buffer overnight at 4° C. The dialysis tubing molecular weight cut off (12-14 KDa) retained the proteins inside the tube and allowed the buffer exchange between the protein solution and the dialysis buffer during all overnight incubation. Considering the difference in volume between protein solution (5 ml) and dialysis buffer (4000 ml), the imidazole present in the protein solution was drastically reduced to less than 1 mM (concentration diluted approximately 800 times).

After removing most of the imidazole, the protein concentration was calculated and 2 mg/ml stocks of protein were generated and stored at -80° C. Based on the number of stocks generated (approximately 20 stocks containing 0.5 ml of 2 mg/ml of protein), protein yields were estimated to be approximately 30-50 mg/l of culture.

3.2.7. Hexahistidine tag detection

Ni-NTA purification is based on the affinity of histidine tags. A further confirmation of the presence of the hexahistidine tag was obtained by performing a protein immunoblot (western blot) using an antibody able to recognize the HisTag only. This was also used to clarify if the two bands obtained for PA0949 were both his-tagged proteins and whether the purification of one of them was due to non-specific binding with the Ni-NTA matrix.

Different recombinant protein and anti-histidine antibody primary and secondary antibody concentrations were tested in order to find the optimum conditions for detection of recombinant proteins. The results show a single band for PA1204, PA2580 and PA2280 and two bands for PA0949 (Figure 3.14), confirming the results obtained during the protein production and purification.

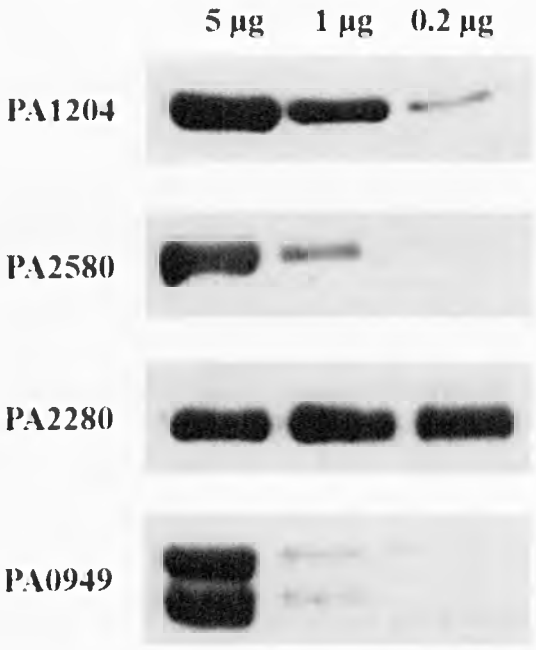


Figure 3.14 Purified putative *P. aeruginosa* PAO1 azoreductase proteins immunoblot. Photographic film sections showing the immunoblotting of PA1204, PA2580, PA2280 and PA0949 using a rabbit primary anti-his (diluted 1:1000 dilutions) and a rabbit secondary (diluted 1:1000) antibodies. One band, indicating the presence of the HisTag, was found for PA1204, PA2580 and PA2280, and two bands for PA0949, indicating the presence of HisTag on each of them. Samples were transferred to the blotting membrane from gels prepared as described in section 2.5.3 using 5, 1 and 0.2 µg of each protein.

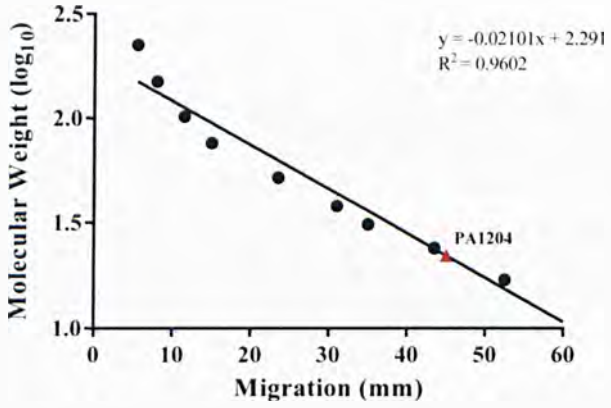
Except for PA2280, for which a strong signal was obtained for all protein concentrations used (probably due to protein dilution issues), there was a correlation between protein concentration and signal intensity. This results indicate that all recombinant proteins purified still have the hexahistidine tag, including the two polypeptides obtained for PA0949.

3.2.8. Protein molecular weight determination

The molecular weight of each protein was determined based on their migration in comparison with the migration of the protein molecular marker bands on SDS-PAGE (Figure 3.15). Protein molecular weights were calculated for small and large scale protein production as well as the Ni-NTA protein purification. The average of three molecular weight measurements was calculated and compared with the predicted molecular weight of each protein (Table 3.8). The predicted molecular weights were calculated based on their amino acid sequences including the tag at the N-terminus, using the protein sequences available on the Pseudomonas Genome Database (Winsor et al., 2011). These were elaborated using ProtParam tool available on ExPASy Bioinformatic Resource Portal (Gasteiger et al., 2005).

The molecular weights obtained were similar to the predicted protein molecular weights. This has been shown in particular for the proteins PA2280 (ΔMW 0.15 KDa) and the upper band of PA0949 (ΔMW 0.44 KDa). A slightly greater difference has been found for PA1204 and PA2580, which were shown to be respectively 1.01 and 1.64 KDa different from the predicted molecular weight. Regarding the lower band of PA0949, it showed the highest difference with the predicted molecular weights of all proteins analysed (ΔMW 3.43 KDa).

a



b

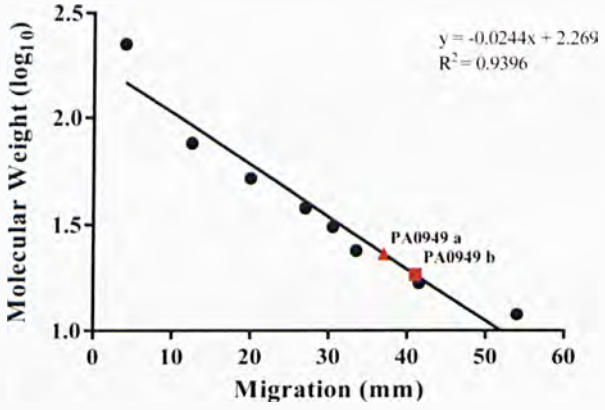


Figure 3.15 PA0949 and PA1204 protein molecular weight estimation.
Graphs showing the $\log_{10}$ protein molecular weights of PA1204 (**a**) and PA0949 (**b**). Log_{10} of the marker molecular weight bands were calculated and associated to the migration (mm) of each band (black circles). Linear regression for all marker bands was calculated and the $\log_{10}$ of the protein molecular weights were obtained based on their migration on SDS-PAGE (red triangles and squares). Graphs generated with GraphPad Prism 6.

Table 3.8 Putative azoreductase protein molecular weights calculated from SDS-PAGE.
Comparison between measured (average of molecular weights calculated from protein production at small and large scale and purification SDS-PAGEs) and predicted protein molecular weights. Protein amino acid sequences were used to calculate the molecular weights on ExPASy Bioinformatic Resource Portal (Gasteiger et al., 2005).

Protein	Measured molecular weight from SDS-PAGE (KDa)	Measured molecular weight from NPL (KDa)	Predicted molecular weight (KDa)	ΔMW (KDa)
PA1204	21.37	22.50	22.38	1.01
PA2580	22.49	24.28	24.13	1.64
PA2280	28.20	28.55	28.35	0.15
PA0949 (upper band)	23.37	22.80	22.93	0.44
PA0949 (lower band)	19.50			3.43

Considering the method limitations (manual measurements of the bands migration) and the thickness of the bands, these results indicate that PA1204, PA2580 and PA2280 proteins can be considered of the correct molecular weight. Regarding PA0949, the upper band molecular weight appears to be similar to the expected molecular weight of this protein, suggesting that this is likely to be the PA0949 target protein.

In order to obtain the accurate molecular weight an aliquot of 50 µl of each purified protein stock (2 mg/ml) were sent to the National Physical Laboratory (NPL) for mass spectrometry analysis. The protein molecular weights obtained (Table 3.8) were highly similar to the predicted molecular weights (ΔMW between 0.2 and 0.12) confirming the hypothesis for which all the proteins purified were of the correct molecular weight.

The mass spectrometry chromatogram analysis showed the presence of a single protein for PA1204, PA2580 and PA2280, confirming the presence of a single polypeptide in each solution. Interestingly, a single protein was also obtained for PA0949, in contrast with what was observed on SDS-PAGEs and western blot at the protein production, purification and HisTag detection stages. For this reason, all the proteins were analysed on SDS-PAGE once more (after one month stored at -80° C) to verify the presence of all bands previously found (one band for PA1204, PA2580, PA2280 and two bands for PA0949). The gel analysis showed a single defined band for all proteins, including PA0949, which shows a single band of approximately 24 kDa (Figure 3.16). This result confirmed those obtained with the mass spectrometry analysis and at the same time contradicted those obtained with the expression, purification and western blot analysis of the original gels of PA0949.

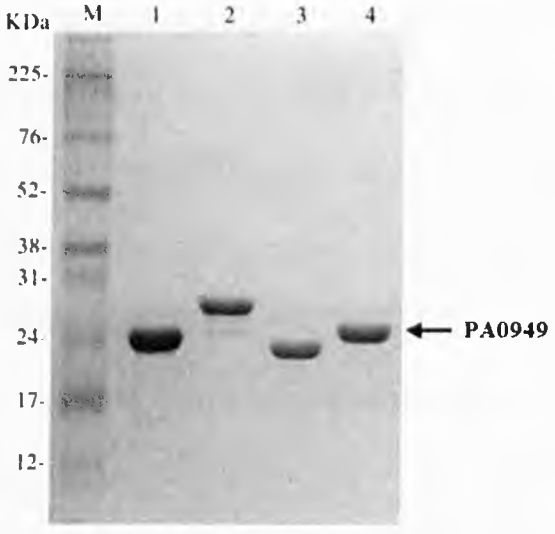


Figure 3.16 Purified proteins SDS-PAGE analysis after storage at -80° C for 1 month. SDS-PAGE showing a single band for the purified putative azoreductase proteins, including PA0949 (black arrow) for which two bands have been previously find (at the expression, purification and western blot proteins analysis). Samples shown are Amersham ECL High-Range Rainbow Molecular Weight Marker (lane M), PA2580 (lane 1), PA2280 (lane 2), PA1204 (lane 3) and PA0949 (lane 4). Ten µl of sample were loaded in each well. Ten µl of samples were loaded in each lane.

3.2.9. Hexahistidine tag cleavage

In order to remove the hexahistidine tags from each protein, an enzymatic reaction was performed using thrombin. Proteins and enzyme were incubated at 4° C for 16 hours, and then analysed on SDS-PAGE. The results shown a reduction of the molecular weight for all proteins (Figure 3.17). A single defined band was obtained for each of the digested protein samples, indicating the complete tag removal. Regarding PA0949, a single defined band was obtained for both digested and undigested samples. This was in contrast with what has been found previously, where two bands were found (protein production, purification and his tag detection), but in line with what has been found with the mass spectrometry analysis.

Molecular weights of digested and undigested proteins were calculated and the differences (ΔMW) are shown in table 3.9. Within the limits and approximations of the technique (manual measurements of the bands migration), the difference between undigested and digested proteins were shown to correspond to the calculated HisTag molecular weight (2.18 KDa) for the protein PA1204 and PA2580 (less than 0.5 KDa different from the tag) and quite similar for PA2280 and PA0949 (approximately 1 KDa different from the tag).

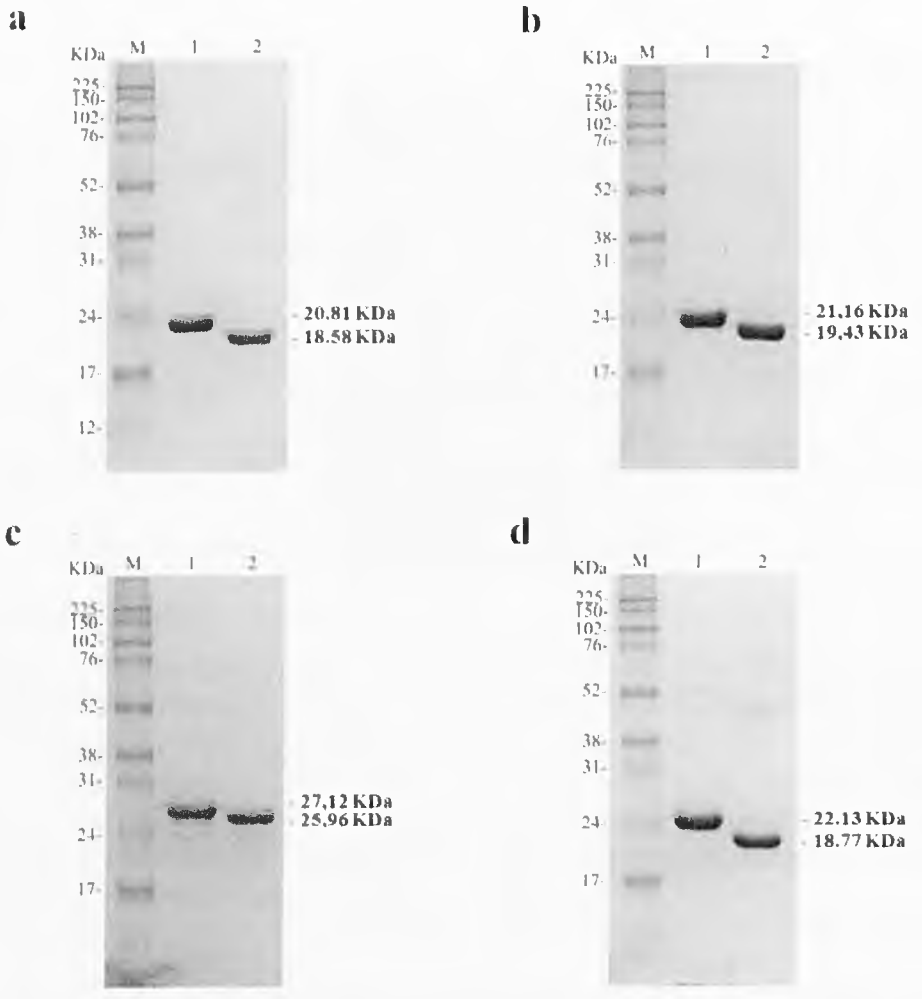


Figure 3.17 SDS-PAGE of thrombin digested and undigested His-tagged putative azoreductase proteins.

SDS-PAGEs showing the molecular weight reduction of thrombin digested samples in comparison with undigested samples of PA1204 (**a**), PA2580 (**b**), PA2280 (**c**) and PA0949 (**d**) purified proteins. Samples used were stored for one month at -80° C before thrombin digestion. Samples shown are Amersham ECL Full-Range Rainbow Molecular Weight Marker (lanes M), undigested sample (lanes 1) and digested sample (lanes 2). Ten µl of samples were loaded in each lane.

Table 3.9 Protein molecular weight before and after thrombin digestion.

Table showing the molecular weights of thrombin treated and untreated putative azoreductase protein samples calculated based on the bands migration on SDS-PAGE and ΔMW.

Protein sample	Measured molecular weight (KDa)	ΔMW (KDa)	Protein tag molecular weight (KDa)
PA0949 undigested	22.13	3.36	2.18
PA0949 digested	18.77		
PA1204 undigested	20.81	2.23	
PA1204 digested	18.58		
PA2280 undigested	27.12	1.16	
PA2280 digested	25.96		
PA2580 undigested	21.16	1.73	
PA2580 digested	19.43		

3.3. Discussion

The aims described in section 1.6 have been met as follow:

Sequencing and cloning of putative *P. aeruginosa* PAO1 azoreductase genes

a. *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975* genes were sequenced, confirming the identity between the genes sequenced and the published database sequences.
b. *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975* genes were amplified via PCR and cloned into *E. coli* JM109ES strain. The cloning was confirmed via PCR and sequencing.
c. Plasmids containing each of the *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975* genes were extracted from *E. coli* JM109ES and transformed into *E. coli* BL21 for recombinant protein production.

Production and purification of putative *P. aeruginosa* PAO1 azoreductases as recombinant proteins

a. Putative *P. aeruginosa* PAO1 PA0949, PA1204, PA2280 and PA2580 recombinant proteins were produced on a small and large scale.
b. The produced recombinant proteins were purified obtaining highly pure his tagged proteins.
c. Recombinant protein molecular weights were determined, confirming the correspondence with the expected molecular weight for each protein.
d. Hexahistidine tags were detected and removed via thrombin digestion.

The availability of the *P. aeruginosa* PAO1 whole genome sequence gives the opportunity to study the molecular and physiological roles of genes for which the function is still unknown. This also increases the understanding of a complex genome, which is the key feature of the adaptation and infectivity of *P. aeruginosa*.

Recombinant DNA methods are a well-established way to manipulate and characterise new genes in order to understand their molecular and physiological roles. The genes *paazor1*, *paazor2* and *paazor3*, which were proposed to encode azoreductases in *P. aeruginosa* PAO1 had already been cloned and expressed using the pET expression system and the corresponding proteins have been characterized as azo-and quinone oxidoreductases (Ryan et al., 2010a, Wang et al., 2007). Further bioinformatics analysis have identified seven new genes in *P. aeruginosa* PAO1 with putative azo- and quinone-oxidoreductase activity (Ryan et al., 2014). Although nucleotide and protein sequences show low homology, structural and functional data of homologue proteins from other organisms suggest that these genes may encode for proteins which display similar function of the known azoreductase proteins from *P. aeruginosa* PAO1.

In order to prove the function of these genes, the corresponding nucleotide sequences were cloned from *P. aeruginosa* PAO1 and prepared for protein production, purification and characterization. Between the seven new identified genes, five were used in this work: *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*. These genes were sequenced before cloning, confirming the correspondence to the sequences available in the Pseudomonas Genome Database for *P. aeruginosa* PAO1 strains (Winsor et al., 2011).

The cloning was successfully performed using the pET system, following a similar protocol already established for *paazor1-3* genes (Wang et al., 2007). The insertion of the correct gene into the vector sequence was monitored by performing PCR reactions using gene specific primers. Following the screening of clones in *E. coli* JM109ES cells, it was possible to determine the size of amplification products by comparing the migration of the

PCR products to those related to the bands of the marker. The amplification with gene specific primers together with a good correlation between expected and calculated sizes indicated a high probability of success of the cloning process. This was finally confirmed by sequencing of each clone, giving the final positive answer.

The plasmid containing the putative azo- and quinone- reductase genes *pa0949*, *pa1204*, *pa2280* and *pa2580* were then used to transform *E. coli* BL21 cells. The clones in this way generated were then used to produce the corresponding recombinant proteins PA0949, PA1204, PA2280 and PA2580 in *E. coli* BL21 strain. As shown with SDS-PAGE, over production of the target proteins was obtained at small scale only for the clone samples (plasmids containing genes cloned) in comparison with negative control samples (empty plasmid).

For the proteins PA1204, PA2280 and PA2580 a single intense band was obtained on SDS-PAGE, indicating the over production of one protein for each gene cloned and expressed, whereas two distinct bands were obtained for PA0949 sample. This raised the question if two different proteins were over produced or if the same protein was truncated generating the two bands observed. Two more protein production assays were performed at small scale for this protein using two different BL21 clones (positive single colony clones isolated and screened) and equivalent results were obtained. This shows the consistency of the presence of two bands instead of one, suggesting that these results did not depend on the specific clone used.

Regarding the protein solubility, all recombinant proteins produced have been shown to be collected mainly in the soluble phase. These results are consistent with those obtained with the known azoreductase proteins from *P. aeruginosa* PAO1 previously characterized (paAzoR1-3) and which shows these proteins to be found between 50 and 60% in the soluble fraction (Ryan et al., 2010a, Wang et al., 2007).

Based on the successful small scale protein production, the process was scaled up

using 500 ml of culture. Following the protein extraction at the larger scale, PA1204, PA2580 and PA0949 were isolated partly in the soluble and partly in the insoluble fraction in a similar fashion to that observed at small scale, whereas PA2280 was entirely isolated in the insoluble fraction. This indicated that an increase of culture volume for PA2280 affected the protein solubility, making the further analysis more complicate. For this reason, PA2280 was over produced using several small scale cultures (100 ml) reproducing the results previously obtained at small scale.

Data related to the *E. coli* WrbA (homologue of the *P. aeruginosa* PAO1 PA0949) shown that FMN molecules added during to the expression medium increase the thermostability of this protein, avoiding the formation of protein aggregates, which are extremely insoluble (Rafii et al., 1990, Chung et al., 1978, Natalello et al., 2007). Although the protein solubility could have been increased for all proteins, PA2280 in particular, using FMN as additional flavin source (flavin molecules bind and stabilize azoreductase proteins, affecting their solubility and thermostability), the SDS-PAGE results indicated no need for the addition of exogenous flavin.

Using the pET system, a hexahistidine tag was included in the sequence of each recombinant protein produced. The presence of polyhistidine tag at the N-terminus of each protein allowed their purification using the IMAC system, which is based on the affinity between a metal ion (nickel) and histidine residues. In this way, only the proteins with the HisTag are bound and kept by the purification matrix (Ni-NTA), allowing the separation from the rest of the protein. The protein elution is then triggered by using elution buffers with high imidazole concentrations. Considering this, the soluble fractions previously obtained were applied to the Ni-NTA columns and pure recombinant proteins were eluted using increasing concentrations of imidazole.

Based on the number of histidine residues (six) present on each protein, these should have been eluted using between 100 and 250 mM of imidazole as observed for the

already characterized paAzoR1-3 from *P. aeruginosa* PAO1 (Wang et al., 2007, Ryan et al., 2010b). However, a wider range of imidazole concentrations (from 10 to 500 mM) were used and all eluates were analysed on SDS-PAGE to identify the fractions containing the proteins of interest. The proteins PA2580, PA2280 and PA0949, were eluted with one or two 250 mM imidazole washes, whereas 500 mM imidazole was needed to elute PA1204. The SDS-PAGE analysis of the purified recombinant proteins showed a single defined band of the approximately correct molecular weight, except for PA0949 which showed the presence of two bands. This indicates that both bands, representing two polypeptides, still have the HisTag in the protein sequence, excluding the possible HisTag loss for part of the protein produced.

After removing most of the imidazole via dialysis, the protein concentration was calculated and 2 mg/ml stocks of protein were generated and stored at -80° C. Protein yields were estimated to be approximately between 30 and 50 mg/l of culture. For a further confirmation of the presence of the HisTag in all purified proteins and the two PA0949 bands, a western blotting analysis was performed using an anti-his antibody. In this case one band was obtained for all protein except for PA0949, where two bands were found, confirming the presence of the HisTag in both polypeptides.

The recombinant protein molecular weights were calculated based on the band migration on SDS-PAGEs and for the proteins produced at small and large scale and purified. The average of the three molecular weights were then calculated and compared with the expected molecular weight. In particular, PA2280 and the upper band of PA0949 showed a good correspondence with a ΔMW of 0.15 and 0.44 KDa respectively. Considering the thickness of the protein bands (sometimes more than 2 mm), which could increase the error degree during the calculation process, these proteins can be assumed of the correct molecular weight.

Regarding PA1204 and PA2580 the ΔMW obtained were 1.01 and 1.64 KDa

respectively. In the light of the level of accuracy of the measurement technique, the possible calculation errors and the fact that sometimes proteins migrate slightly different from their expected molecular weight, PA2580 and PA1204 can also be assumed of the correct molecular weight. An example of a similar case is represented by the paAzoR1 protein for which the molecular weight has been calculated from SDS-PAGE to 30 KDa against the predicted molecular weight of 25 KDa. In this case, even with a five KDa difference, the protein has been shown to be correct (Wang et al., 2007).

Regarding the PA0949 lower band molecular weight (19.50 KDa with ΔMW of 3.43) it is compatible with anomalous protein migration on SDS-PAGE (sometimes proteins migrate slightly different from their expected molecular weight), however the presence of two bands leads to the hypothesis of the presence of two polypeptide chains. Based on this, the PA0949 lower band could have been obtained after a protein truncation, for example at the C-terminus, as the HisTag analysis (HisTag present at the N-terminus only) shown two distinct proteins.

In order to establish the real molecular weight of each produced and purified recombinant protein, mass spectrometry was performed. The obtained molecular weights were 22.50 KDa (PA1204), 24.28 KDa (PA2580), 28.55 KDa (PA2280) and 22.80 KDa (PA0949), indicating the correspondence with the predicted molecular weight (ΔMW between 0.12 and 0.2 KDa only). The analysis of mass spectrometry chromatograms showed the presence of a single species in the solution for all proteins analysed, including PA0949. For this protein the molecular weight obtained with mass spectrometry corresponded to the band of higher molecular weight on SDS-PAGE, which was the closest to the predicted value. As this was in contrast with what has been previously found on SDS-PAGE during the protein production and purification, a new SDS-PAGE was run showing only one band of approximately 24 KDa. It is proposed that the lower molecular weight band had been extensively hydrolysed during the storage process despite the

presence of protease inhibitors and the low temperature. During the heterologous protein induction, the activity of strong promoters or the high inducer concentration can lead to an extremely high protein concentration inside the cells. This can cause further formation of prematurely terminated peptides, incorrect and/or partially folded proteins, explain the presence of two bands on the SDS-PAGE (Baneyx and Mujacic, 2004). If the PA0949 lower molecular weight band was partially synthetized or incorrectly folded, its hydrolysis or precipitation during the freeze-throw process is a very strong possibility.

The protein tags incorporated at the N-terminus of each protein, included a hexahistidine tag as well as the thrombin cleave site. This is normally used to cleave the tag from the native protein amino acid sequence after the purification process. This was performed on all purified proteins PA0949, PA1204, PA2280 and PA2580. Molecular weight reduction was observed for the digested samples when they were compared with undigested samples on SDS-PAGE. The presence of single defined bands for the digested proteins indicated the complete tag removal from each protein. One band was also obtained for both digested and undigested PA0949 samples. This was in contrast with the protein production, purification and HisTag detection, where two bands had been shown. However, this was in line with what has been found with the mass spectrometry analysis, suggesting that a single polypeptide was now present in this solution.

Having generated the pure recombinant proteins, the following chapter describes the characterisation of these protein in terms of their physical, chemical and enzymatic properties.

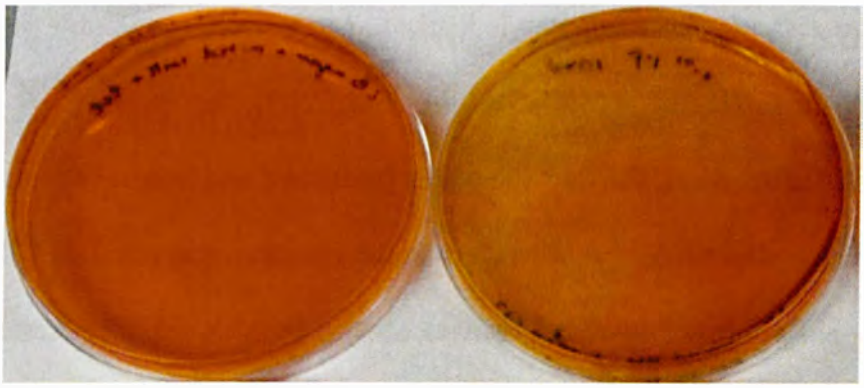
Chapter 4. Characterization of *P. aeruginosa* PAO1 putative azoreductases

4.1. Introduction

Azoreductases are a family of enzymes that have been found in many bacterial and fungal species, including *E. coli*, *Bacillus subtilis*, *Enterococcus faecalis*, *Rhodobacter sphaeroides*, *P. aeruginosa*, *Sinorhizobium meliloti*, *Staphylococcus aureus*, *Saccharomyces* as well as in humans (Ye et al., 2007, Sollner et al., 2007, Cui et al., 1995, Nakanishi et al., 2001, Sugiura et al., 2006, Liu et al., 2007, Chen et al., 2004, Wang et al., 2007, Nachiyar and Rajakumar, 2005, Chen et al., 2005b).

The *P. aeruginosa* azoreductases 1, 2 and 3 (paAzoR1-3) have been described as catalysing the reduction of an azo bond (-N=N-) in azo pro-drugs and azo dyes such as methyl red (Figure 4.1) (Wang et al., 2007, Ryan et al., 2010a). The reaction mechanism of azoreduction by these enzymes has been proposed and it shows that the reduction of azo substrates follows a characteristic ping pong bi bi mechanism (Wang et al., 2010). As described in chapter 1, the mechanism requires two cycles of reduction of flavin (e.g. from FMN to $FMNH_2$), which reduces the azo substrate to a hydrazine in the first cycle, and reduces the hydrazine to two amines in the second cycle (section 1.6, Figure 1.4). This indicates that the presence of flavin groups is essential for the reaction. Although not all azoreductase enzymes require flavin for their activity (Chen et al., 2010a), the previous observation is also supported by the fact that many enzymes showing azoreductase activity all share a common core flavodoxin-like fold (Binter et al., 2009, Wang et al., 2007, Ito et al., 2006, Sanders et al., 2006, Faig et al., 2000). The reduction of flavin is carried out by electron transfer from the electron donor NAD(P)H. Since one molecule of NAD(P)H + H^+ is a two-electron carrier and the reduction of one molecule of azo substrate requires four electrons, two molecules of NAD(P)H are required to complete the reaction. For this

reason the azo reduction of substrates is likely to correspond to a double ping pong bi bi reaction mechanism (Figure 4.2) (Nakanishi et al., 2001, Ito et al., 2006, Wang et al., 2007). During the reaction, the two substrates (reductant and azo substrate) in turn enter inside the active site while the flavin co-factor remains non-covalently bound to the protein. The ping pong bi bi reaction, also known as a double displacement reaction, applies where the two substrates cannot bind the protein at the same time, and therefore the binding of the azo substrate occurs when NAD(P)+ leaves the enzyme active site (Cleland, 1963, Yang et al., 2005).



Control (no bacteria) ***P. aeruginosa*** **PAO1**

Figure 4.1 Methyl red decolourization by *P. aeruginosa* PAO1.
Degradation of the azo dye methyl red by *P. aeruginosa* PAO1 on LB agar with 50 µM dye. The methyl red, normally dark orange, turns yellow when reduced by *P. aeruginosa* azoreductase enzymes. Plate inoculated with 50 µl of *P. aeruginosa* PAO1 culture was compared with not inoculated plate (Control).

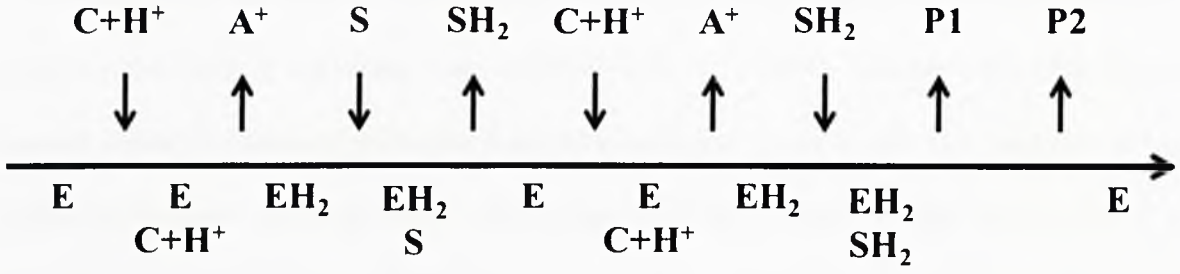


Figure 4.2 paAzoR reaction mechnism.
Diagram representing the ping pong bi bi mechanisms. The substrates are bound to the active site one at time. Two NAD(P)H molecules (two electrons each) are needed to reduce the azo substrate to two aromatic amines. E=enzyme; $C+H^+$=NAD(P)H+H^+; $EC+H^+$=enzyme+NAD(P)H+H^+; A^+=NAD(P)$^+$; EH_2=reduced enzyme; S=azo substrate; EH_2S=reduced enzyme+azosubstrate; SH_2=intermediate product; EH_2SH_2=reduced enzyme+intermediate product; P1 and P2= two aromatic amines.

Structural and dissociative data on paAzoR1 has shown that tetramers of this enzyme contain four molecules of non-covalently bound flavin (Wang et al., 2007). These data indicate that a flavin molecule is required for the enzymatic reduction of substrates: hence azoreductases fall into the category of enzymes known as flavoenzymes.

The ability of azoreductases to reduce a variety of both endogenous and exogenous compounds has complicated the identification of their physiological substrate and hence their function. Some azoreductases have been shown to reduce quinones, which are normally reduced by a group of NAD(P)H quinone oxidoreductases (NQOs) (Binter et al., 2009, Nakanishi et al., 2001, Chen et al., 2004, Liu et al., 2009a). The quinone oxidoreductase group of enzymes has also been found in many species from bacteria to mammals and have previously been considered a distinct group from the azoreductases (Nakanishi et al., 2001, Mendes et al., 2011b, Chen et al., 2005a, Wu et al., 1997).

The reduction of quinones occurs via a two-electron transfer reaction from NAD(P)H molecules to the substrate, which is converted to the more stable and less toxic quinol (Chesis et al., 1984). This suggests that hydride transfer by azoreductases would also be possible to a quinone substrate (Ryan et al., 2010a). This hypothesis was tested and confirmed by Ryan et al., showing that paAzoR1-3 enzymes are able to reduce quinone substrates as well as azo compounds (Ryan et al., 2014).

The three azoreductases from *P. aeruginosa* PAO1 were initially identified via sequence homology to *E. coli* AzoR enzyme using a 30% homology cut-off, because of the low sequence identity (Wang et al., 2007). The low sequence homology across azoreductase enzymes makes their identification difficult based purely on amino acid sequence similarity.

In a recent study, a combination of bioinformatics approaches was applied for the identification of new putative azoreductase enzymes in *P. aeruginosa* PAO1. In particular,

structural and experimental data of known azo- and quinone- reductases from many species were collected and used to search for new enzymes in *P. aeruginosa* PAO1. As a consequence seven new putative azoreductases were identified (Figure 4.3), including PA0949, PA1204, PA1224, PA1225, PA2280, PA2580 and PA4975 (Ryan et al., 2014).

It is evident from a comparison of protein sequences of these enzymes that they show limited similarity even though they may have a similar enzymatic function. PA2580, for example, shares significant homology with a group of enzymes known as Modulator of drug activity B (MdaB) (Chatterjee and Sternberg, 1995). The structure of *E. coli* MdaB (75% similarity with PA2580) shows to be similar to paAzoR1 and also exhibits an NAD(P)H quinone oxidoreductase activity (Hayashi et al., 1996). Similar results have also been found for the MdaB from *Helicobacter pylori* (Wang and Maier, 2004).

Structural data is also available for the protein PA1204 and it too shows high similarity with paAzoR1 and its closest homologue from *A. thaliana* (69% homology) has been shown to have NAD(P)H quinone oxidoreductase activity (Agarwal et al., 2006, Sparla et al., 1999).

The crystal structure of PA0949 has been solved and shows a similar structure to paAzoR1 (Gorman and Shapiro, 2005). Additionally, the homologue protein in *E. coli* (56% homology with PA0949) has also been shown to have NAD(P)H quinone oxidoreductase activity (Patridge and Ferry, 2006).

PA2280 was predicted to be part of the ArsH operon, which was initially identified as part of the arsenic resistance operon of *Yersiniae enterocolitica* (Neyt et al., 1997). The homologous proteins in *S. meliloti* (82% homology) and *Synechocistis* sp PCC 6803 (78% homology) have been shown to reduce azo dyes and quinones (Ye et al., 2007, Hervas et al., 2012).

In this chapter the biochemical and enzymatic characterization of the purified putative azoreductase proteins is described.

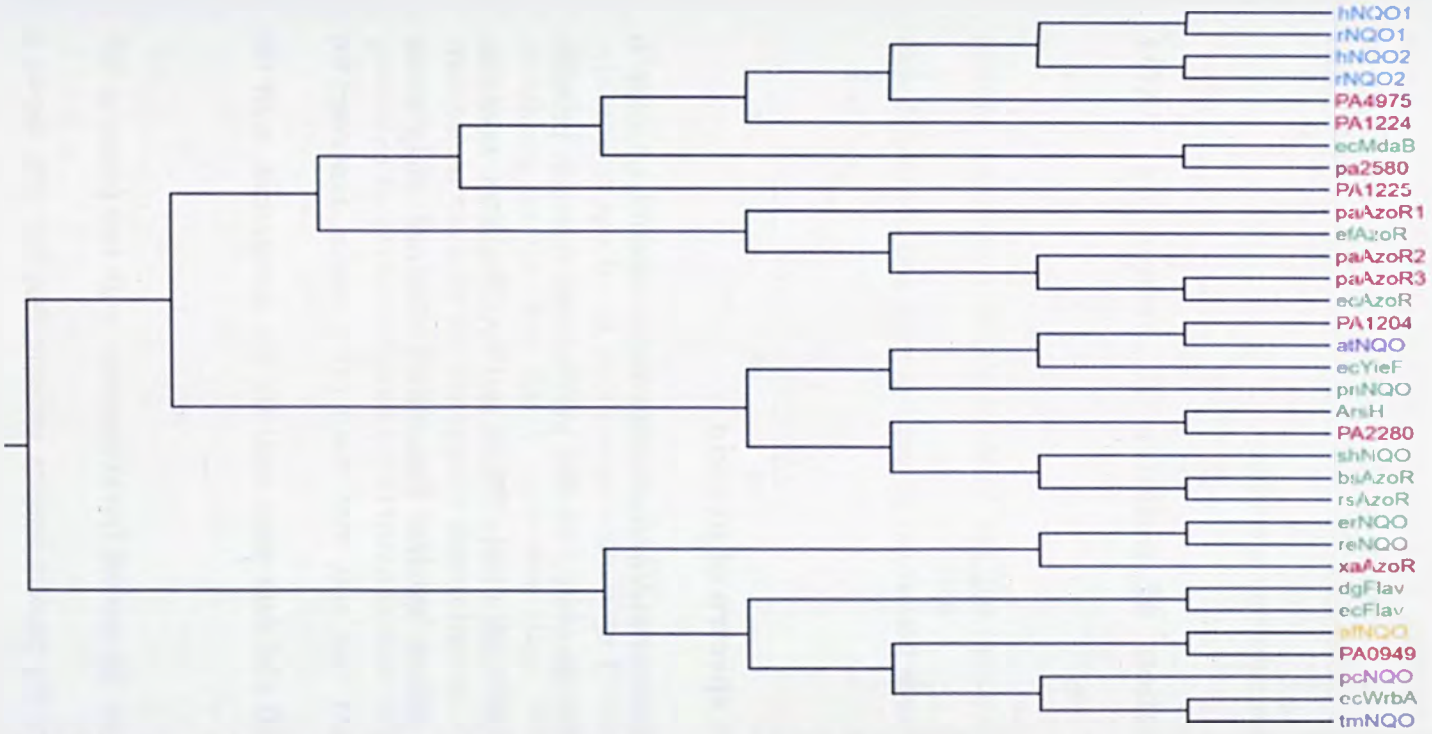


Figure 4.3 Phylogenetic tree showing the relationship between known and putative azoreductases from *P. aeruginosa* PAO1.
Phylogenetic tree illustrating the relationship between enzymes from *P. aeruginosa* (red), other bacterial (green), mammalian (blue), plant (purple), fungi (pink) and archea (yellow). PA0949, PA1204, PA1224, PA1225, PA2280, PA2580, and PA4975 are proteins from *P. aeruginosa* PAO1. ecAzoR, bsAzoR, efAzoR and rsAzoR are azoreductases from *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalus* and *Rhodobacter sphaeroides*. hNQO1 hNQO2, rNQO1 and rNQO2 are human and rat azoreductases. xaAzoR is a flavin independent azoreductase from *X. azovorans*. ecMdaB, ecYieF and ecWrbA are NAD(P)H quinone oxidoreductases from *E. coli*. afNQO, pnNQO, tmNQO, pcNQO and atNQO are NAD(P)H quinone oxidoreductases from *Archaeoglobus fulgidus*, *Paracoccus denitrificans*, *Triticum monococcum*, *Phanerochaete chrysosporium* and *Arabidopsis thaliana* respectively. ArsH is an azoreductase from *Sinorhizobium meliloti*. dgFlav and ecFlav are flavodoxins from *Desulfovibrio gigas* and *E. coli* respectively. shNQO, reNQO and erNQO are uncharacterised proteins from *Staphylococcus haemolyticus*, *Ralstonia eutropha* and *Erwinia chrysanthemi*. Figure taken from Ryan et al. (2014).

4.2. Results

A series of biochemical analysis were performed on the purified *P. aeruginosa* PAO1 putative azoreductase proteins PA0949, PA1204, PA2280 and PA2580 in order to establish the presence of FMN or FAD molecules, the preference of flavin and NAD(P)H during the enzymatic reduction and their substrate specificity.

4.2.1. Flavin group determination of putative *P. aeruginosa* PAO1 azoreductase proteins

In order to determine the presence and the kind of flavin molecules, protein absorption spectra and TLC analysis were performed on each protein and compared with free FMN and FAD molecules.

4.2.1.1. Optical absorbance spectra of protein

In order to determine the presence of Flavin molecules within proteins, absorption spectra between 300 and 600 nm were generated for the purified recombinant proteins PA1204, PA0949, PA2280 and PA2580. Free Flavin (FMN and FAD) spectra were also generated for comparison with the protein spectra. These were obtained using flavin solutions of 10 and 20 μM in 20 mM Tris 100, mM NaCl pH 8 buffer, however, for purpose of case of presentation the 20 μM data were used for the comparison with the protein spectra.

The FMN and FAD results show the typical flavin spectrum with two peaks at 365 and 450 nm (Figure 4.4). Analysis of the protein spectra indicate that PA1204 shows a spectrum similar to the flavin groups (Figure 4.4 **a**) with an absorption shoulder at 450 nm and a less evident one at 365 nm. Although the absorbance peaks for the proteins PA2580 (Figure 4.4 **b**) and PA2280 (Figure 4.4 **c**) were not defined as for PA1204, they were still visible. PA0949 (Figure 4.4 **d**) shows no peaks at 365 and 450 nm, indicating the poor content of flavin for this protein.

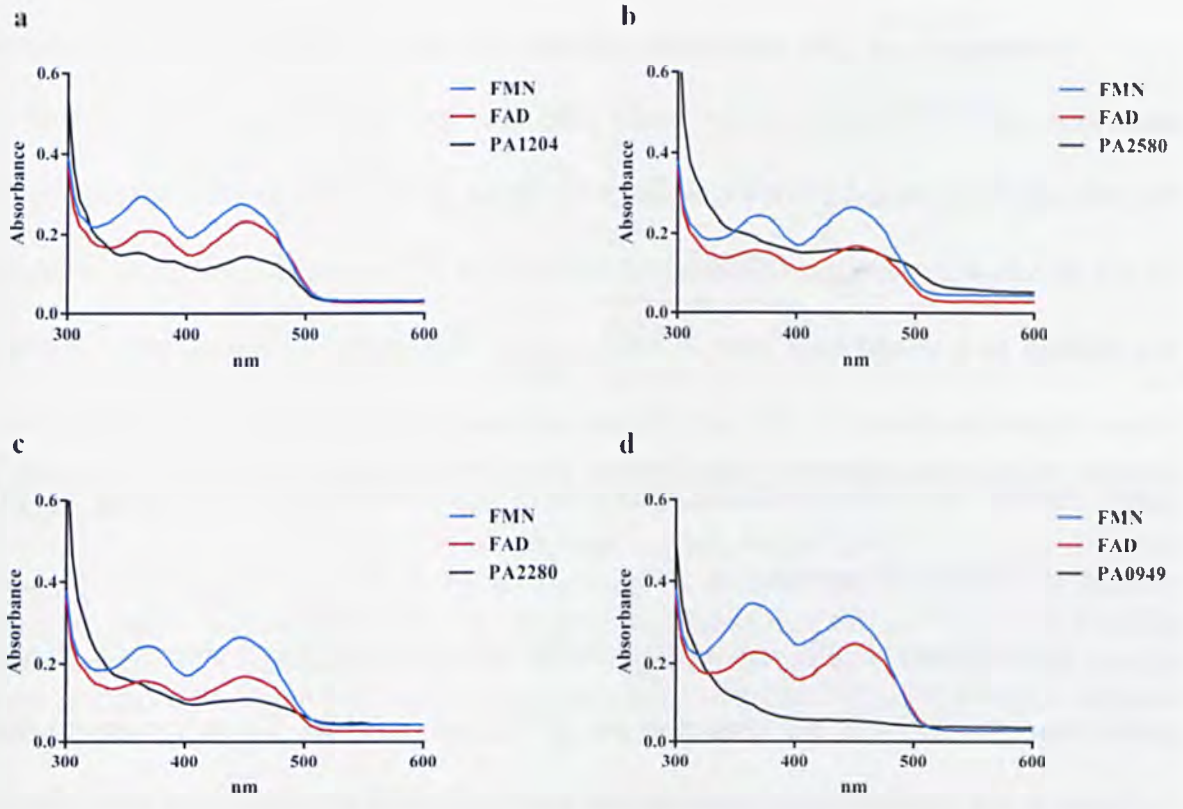


Figure 4.4 Putative *P. aeruginosa* PAO1 azoreductase proteins absorption spectra. Absorption spectra of putative azoreductases PA1204 (**a**), PA2580 (**b**), PA2280 (**c**) and PA0949 (**d**) compared with FMN and FAD absorption spectra, showing two absorption shoulders for the free flavin molecules (λ_{max} 365 and 450 nm). One mg/ml protein solutions were prepared in 20 mM Tris 100 mM NaCl pH 8 buffer and placed in UV transparent cuvettes for the spectra determination. 20 µM FMN and FAD solutions were prepared in the same buffer and analysed in the same way. Absorption spectra were generated for three replicates (n=3) using Infinite M200 PRO plate reader between 300 and 600 nm.

Comparison of the absorption spectra for each protein shows the highest absorbance level at the two flavin peaks (365 and 450 nm) for PA2580, followed by PA1204 and PA2280 and PA0949 (Figure 4.5). These data indicate that PA2580 appears to be the protein with the highest content of flavin, even if the specific absorption peaks are not defined as it would have been expected as the characterized azoreductase 1,2 and 3 shown higher absorbance at 365 and 450 nm indicating higher flavin content (Wang et al., 2007). PA0949 shows the lowest absorbance level between all proteins, indicating the poor content of flavin for this protein.

Although the absorbance at 450 and 365 nm is greater than background, distinct peaks were not found at the concentration of this protein used. There appears to be a variation in the expected molar ratio protein:flavin (280:450 nm absorbance ratio) despite the fact that the proteins were each prepared in the same way. Althoungh the protein:flavin ratio was expected to be one, actual ratio varied from 3:1 (PA2580) to 6:1 (PA0949), confirming the poor intensity of flavin peaks and the flavin content in each protein (PA2580>PA1204>PA2280>PA0949).

4.2.1.2. Thin Layer Chromatography

Whilst no distinct peaks across the flavin region were observed for two of the pure proteins (PA0949 and PA2280), all of the proteins which were eluted from the Ni-NTA purification column were yellow in colour, indicating the presence of flavin molecules (concentrated flavin is yellow). Additionally, the absorption spectra of PA1204 in particular which had the lightest yellow intensity, showed similar absorption peaks to those of free FMN and FAD. These data are consistent with, but do not prove, that the proteins are flavoproteins.

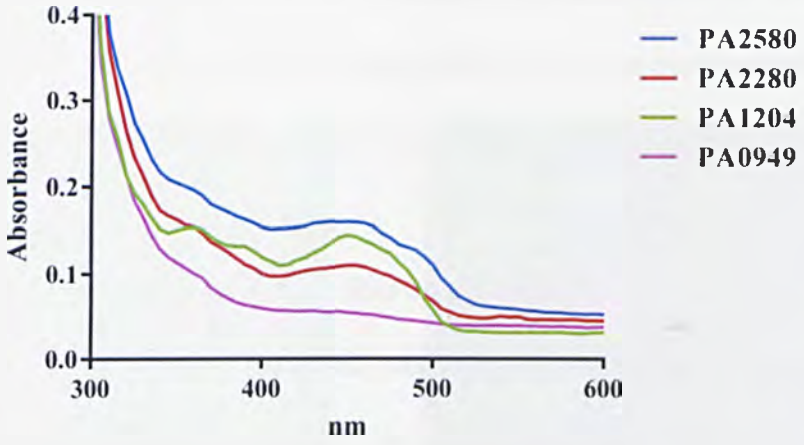


Figure 4.5 Putative *P. aeruginosa* PAO1 azoreductase proteins absorption spectra comparison.

Comparison of absorption spectra of putative azoreductases PA1204 (green), PA2580 (blue), PA2280 (red) and PA0949 (pink) showing the different absorbance at 365 and 450 nm. One mg/ml protein solutions were prepared in 20 mM Tris 100 mM NaCl pH 8 buffer and placed in UV transparent cuvettes for the spectra determination. Absorption spectra were generated for three replicates (n=3) using Infinite M200 PRO plate reader between 300 and 600 nm.

As flavin molecules can be separated on TLC plates due to the difference in polarity, all proteins were run on TLC plates and compared with free FMN and FAD. This was first optimized for the flavins themselves in order to find the optimum concentration to obtain defined spots to use as references.

Four flavin concentrations were tested showing that 0.5 mM was the most appropriate to use as flavin spots were more defined (Figure 4.6). All purified proteins were concentrated to 20 mg/ml and spotted on TLC plates with FMN and FAD in separate lanes. The results show that for PA1204 and PA2580 a visible spot similar to FAD was obtained (Figure 4.7 **a** and **b**). The PA2580 spot was more intense than PA1204, confirming the absorption spectra results, where PA2580 was found the richest in flavin. No spots were observed for PA2280 and PA0949 (Figure 4.7 **c** and **d**), confirming also in this case the spectra result, where really low absorbance was observed at 365 and 450 nm.

Retention factors (R_f) were calculated for PA2580 and PA1204 and compared with those obtained for FMN and FAD (Table 4.1). The PA2580 R_f (0.26) is the same as FAD, indicating that this is the flavin present in this protein. For PA1204, the R_f (0.30) is different from both FMN and FAD. However it is closer to FAD (Δ_{Rf} 0.26) than FMN (Δ_{Rf} 0.039), suggesting that FAD may be flavin also for this protein. No R_f values were calculated for PA2280 and PA0949 because of the absence of spots. Based on these results as well as those obtained with spectra analysis, PA1204 and PA2580 have been shown to be flavoproteins whereas it has not been confirmed by these methods that PA2280 and PA0949 are flavoproteins although they have absorbances in the flavin region which are above background and do show a yellow colour. In addition, information in relation to the role of flavins in enzymic activity (see below) do point to the latter being flavoproteins.

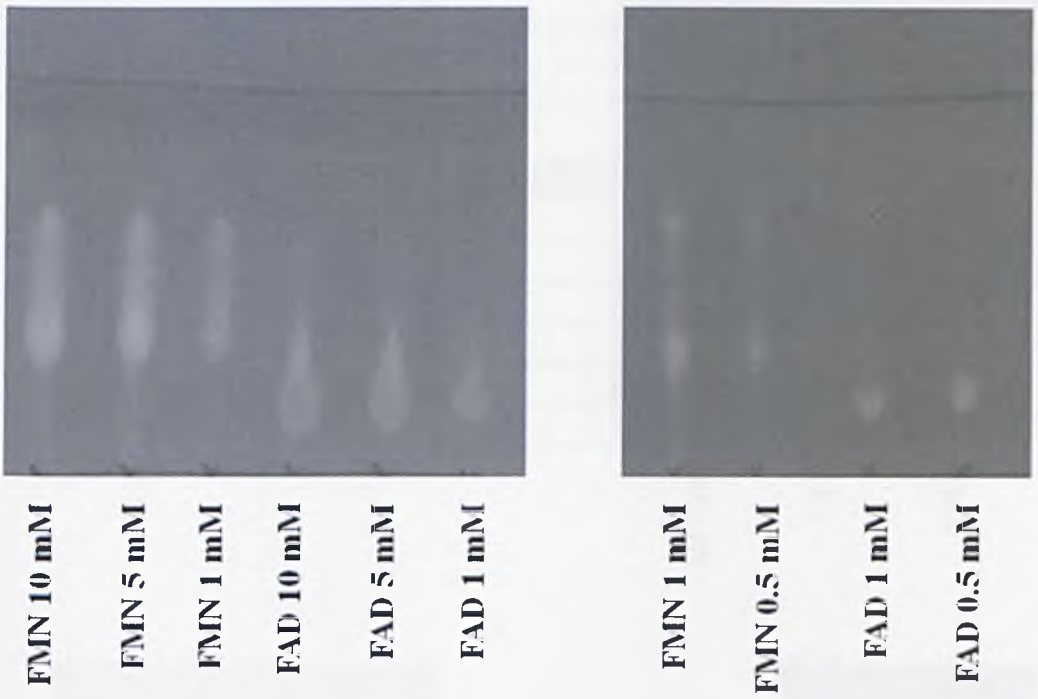


Figure 4.6 TLC optimization of free FMN and FAD.
Thin Layer Chromatography analysis of FMN and FAD on silica plates showing the migrations of the two flavins. Optimization was performed using 10, 5, 1 and 0.5 mM of free flavins prepared in 20 mM TrisHCl 100 mM NaCl pH 8 buffer. N-butanol, acetic acid and water (2:1:1) was used as solvent.

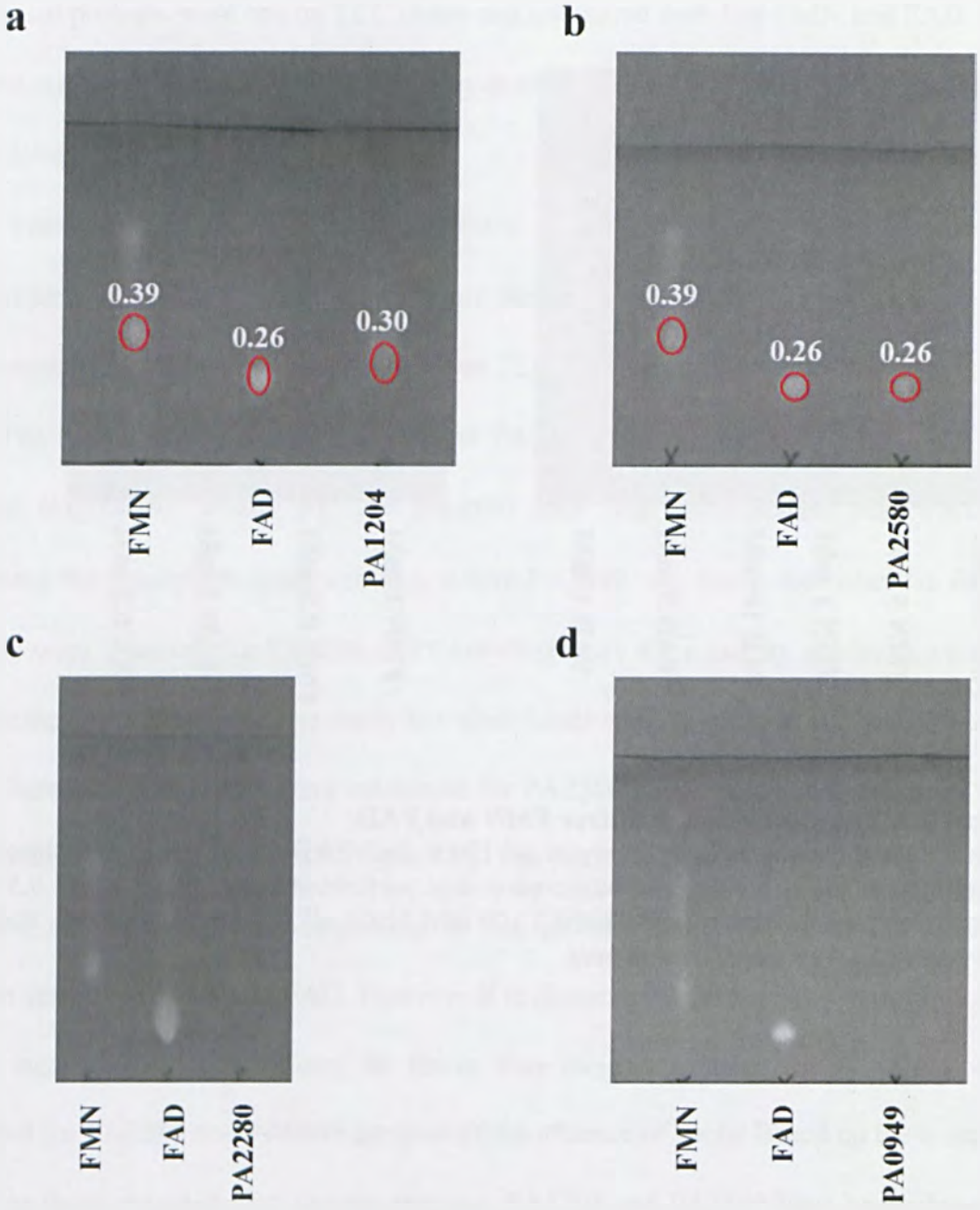


Figure 4.7 TLC profiles of putative *P. aeruginosa* PAO1 azoreductase protein in comparison with free FMN and FAD.

Comparison of thin Layer Chromatography analysis of putative azoreductase proteins, FMN and FAD showing the migrations of each protein in comparison with free flavins. Single spot was found for PA1204 (**a**) and PA2580 (**b**) showing their similarity with FAD (R_f: PA1204 0.30; PA2580 0.26; FMN 0.39; FAD 0.26). No spots were found for PA2280 (**c**) and PA0949 (**d**). Protein and falvin solutions used (20 mg/ml and 0.5 mM respectively) were prepared in 20 mM TrisHCl 100 mM NaCl pH 8 buffer. N-butanol, acetic acid and water (2:1:1) was used as solvent. 1 µl of each solution was used.

Table 4.1 Putative *P. aeruginosa* PAO1 azoreductase proteins and flavins retention factors.

Retention factors (R_f) obtained for FMN, FAD, PA0949, PA1204, PA2280 and PA2580 on TLC plate showing the correspondence between PA2580 and FAD, and the similarity between PA1204 and FAD again. No data are available for PA2280 and PA0949 because the absence of spots on TLC plate. R_f were calculated based on the migration of the compound divided by the migration of the solvent used.

Flavin or protein	R_f
FMN	0.39
FAD	0.26
PA0949	-
PA1204	0.30
PA2280	-
PA2580	0.26

4.2.2. Enzymatic activity of putative *P. aeruginosa* PAO1 azoredutase proteins

To verify whether the putative azoreductase proteins were active or not and to establish the enzymatic reaction conditions in relation to co-factors preference, a series of enzymatic analyses were performed. This was carried out using azo or quinone substrates and comparing the reduction of these substrates in different conditions.

The determination of the enzymatic activity was obtained following the reduction of substrates (for azocompounds) or the oxidation of NAD(P)H by measuring the absorbance at 340 nm (for quinones) during the reaction. Previous data shows that azocompounds absorb at different wavelengths from NAD(P)H, whereas quinones absorb at similar wavelength. It was also shown that the reduction of azocompound requires two NAD(P)H, whereas the quinones require only one NAD(P)H. For these reasons the reduction of azocompound was followed by monitoring their absorbance and for the quinones following the NAD(P)H absorbance.

In order to follow the absorbance changes during the reaction it was important to determine the maximum absorbance (λ_{max}) of the molecules to monitor (azo compound or NAD(P)H). To do this, absorption spectra were generated for all substrates used as well as NAD(P)H.

4.2.2.1. Establish enzymatic assays

4.2.2.1.1. Calculation of molar extinction coefficient

In order to determine the wavelength of maximum absorbance for substrates and NAD(P)H, absorption spectra were generated (Figure 4.8). These data show that all azo compounds tested absorbed at detectable wavelengths (between 300 and 800 nm), whereas the quinones absorbed below 300 nm. For the quinones, absorbance of NAD(P)H, was followed as indication of reduction of these substrates.

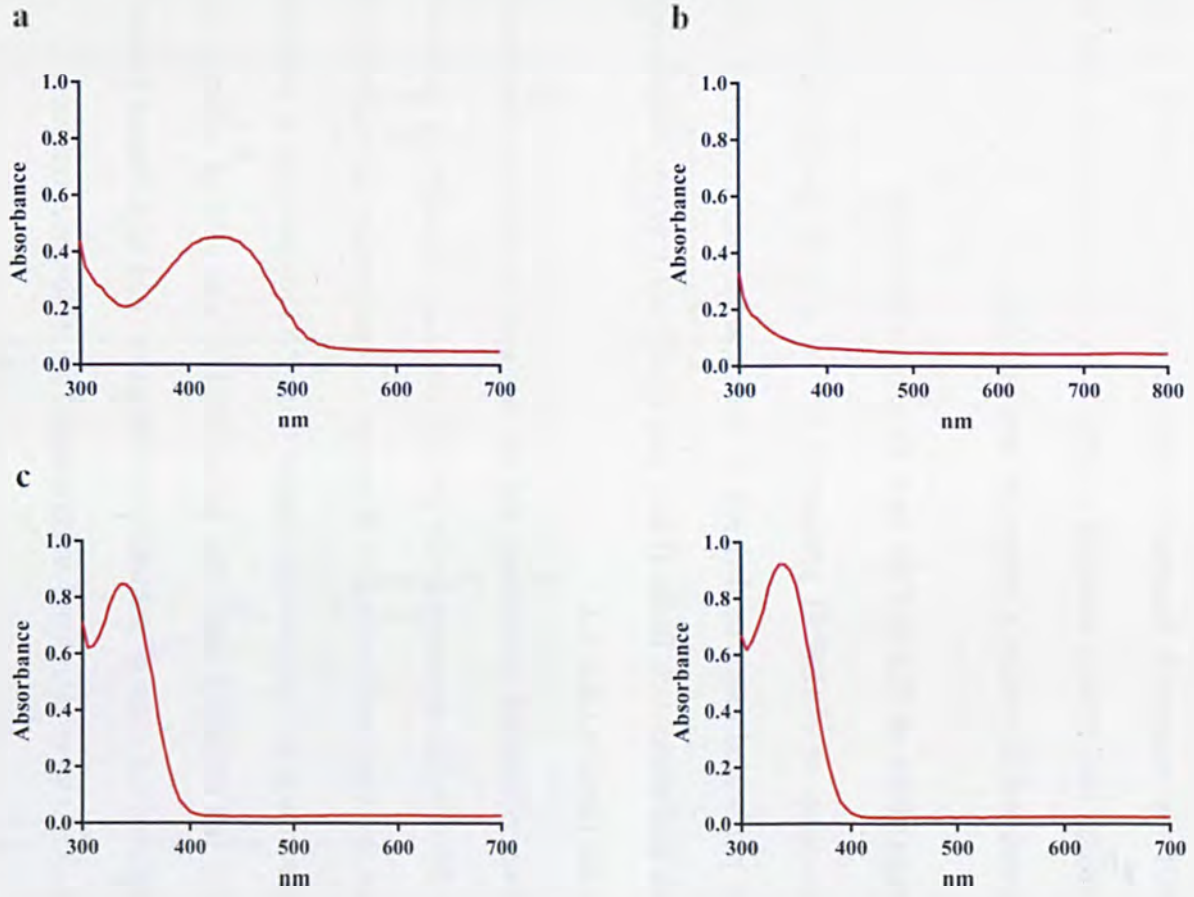


Figure 4.8 Absorption spectra of methyl red (azo compound) (a), Plumbagin (quinone) (b), NADH (c) and NADPH (d).
Absorption spectra of two example of substrates (methyl red and plumbagin) and NAD(P)H co-factors showing λ_{max} of 430 for methyl red (**a**) and 340 nm for NAD(P)H (**c** and **d**). Plumbagin, as the majority of the quinones, does not show any considerable absorbtion level at the wavelength tested. 50 μM of each compound and NAD(P)H were used and prepared in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

The maximum wavelengths obtained for azo compounds and for NAD(P)H were used to calculate the molar extinction coefficient (ε) needed for the calculation of the reduction rates of each enzyme. To do this, the absorbance of increasing concentrations of substrates and NAD(P)H was measured. Regression analysis was then performed on the obtained absorbance values and ε were obtained (Figure 4.9). The compounds used to generate absorption spectra and the related ε values are listed in table 4.2.

4.2.2.1.2. Determination of NAD(P)H and flavin preference

For the determination of NAD(P)H preference and to test the protein activity, enzymatic assays were performed using methyl red as the substrate and NAD(P)H. The assay was also run with and without co-factor (FMN and FAD). All reaction components and combinations used are listed in table 4.3.

The reactions were performed in triplicate and the decrease in substrate absorbance was monitored every 15 seconds measuring the methyl red λ_{max}. Profiles of substrate reduction were obtained showing the decrease of absorbance, representing the reduction of the substrate, while the reaction was proceeding (Figure 4.10, PA2580 protein). It was also observed that the methyl red reduction improved when NADPH was used as cofactor. To compare reaction conditions, the rates of reduction of substrates (μM of substrate reduced per second per mg of protein) were calculated and compared for different conditions.

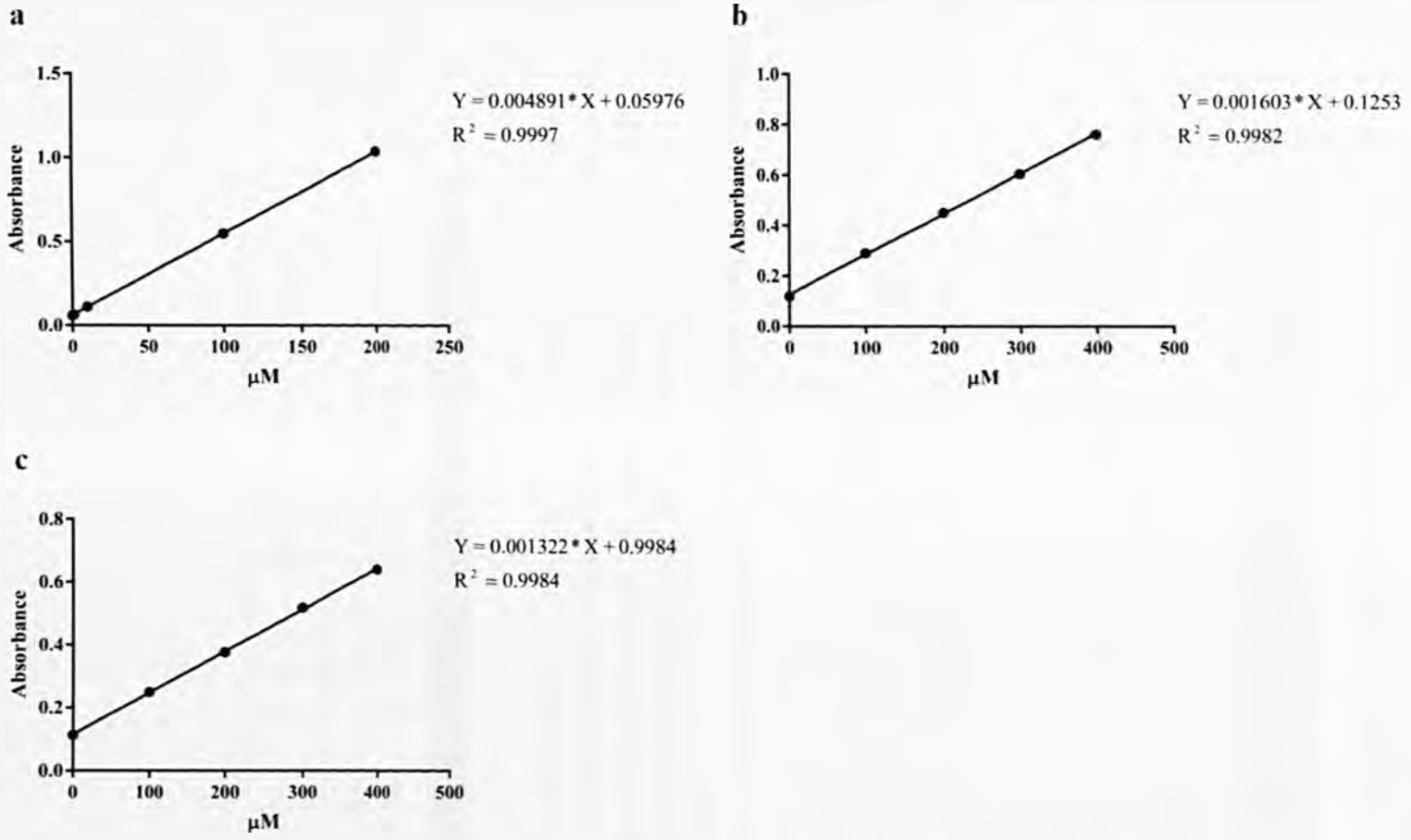


Figure 4.9 Determination of molar extinction coefficient for Methyl red (a), NADH (b) and NADPH (c).
Graphs showing molar extinction factor calculation using different concentration (μM) of methyl red (**a**), NADH (b) and NADPH (c). Absorbance values were obtained measuring each compound/cofactor using their specific λ_{max}. Linear regression was then applied and molar extinction factors were generated.

Table 4.2 Azo componds and NAD(P)H λ_{max} and epsilon values.
Absorbance maximum obtained for azo compouns and NAD(P)H from the absorption spectra analysis and related molar extinction factor values generated for each of them. 50 μM solutions were used and absorption spectra were generated in UV transparent cuvettes between 300 and 800 nm using Infinite M200 PRO plate reader.

Azo compuond	Absorbance peak (λ_{max}, nm)	Molar extinction factor ($M^{-1}cm^{-1}$)
Amaranth	520	$3.16 \cdot 10^{-3}$
Balsalazinde	440	$5.08 \cdot 10^{-3}$
Methyl red	430	$5.25 \cdot 10^{-3}$
Orange G	480	$2.35 \cdot 10^{-3}$
Orange II	485	$3.52 \cdot 10^{-3}$
Ponceau S	515	$3.47 \cdot 10^{-3}$
Ponceau BS	505	$6.30 \cdot 10^{-3}$
Sudan I	530	$1.14 \cdot 10^{-3}$
Tropaeolin	430	$8.59 \cdot 10^{-3}$
NADH	340	$1.60 \cdot 10^{-3}$
NADPH	340	$1.32 \cdot 10^{-3}$

Table 4.3 Enzymatic reaction component combinations used to test protein activity and NAD(P)H preference.
Five combinations of reaction components used in order to establish whether the protein was active as well as the preference in using NADH or NADPH. Reaction 4 and 5 represent the negative controls. Samples were prepared using 10 μg of protein, 50 μM of substrate and 500 μM of NAD(P)H in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

Enzymatic reaction	Protein	Substrate	NADPH	NADH
1	X	X	X	
2	X	X		X
3	X	X		
4		X	X	
5		X		X

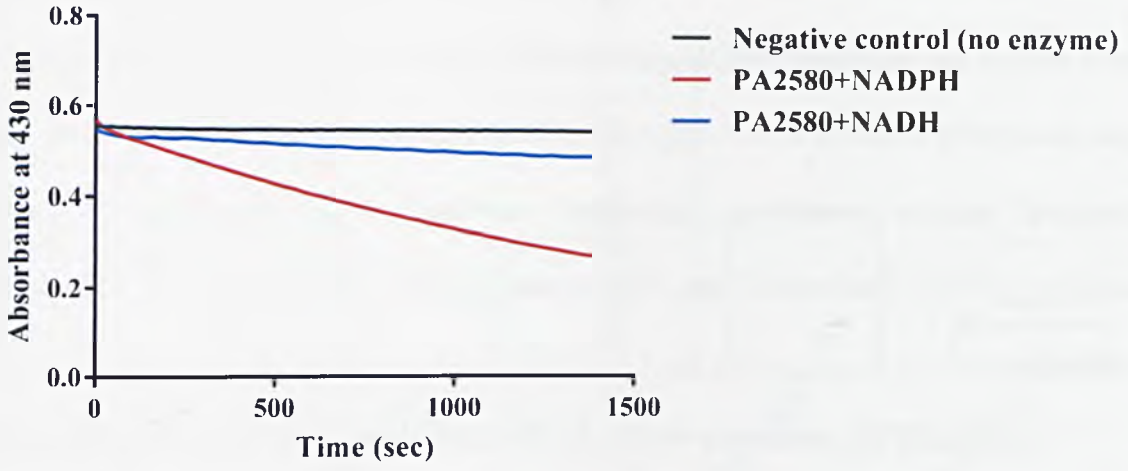


Figure 4.10 Rate of methyl red reduction by PA2580 with and without NAD(P)H. Graph showing the methyl red absorbance measurements during its reduction by PA2580 using NADH (blue line), NADPH (red line) compared with negative control (black line, no protein). The absorbance levels, representing the reduction of methyl red, indicate better reduction when NADPH is used compare with NADH. No absorbance changes were observed for control sample. Reactions were prepared using 1 µg/ml of protein, 50 µM of substrate and 500 µM of NAD(P)H in 20 mM TrisHCl 100 mM NaCl pH 8 buffer in a final volume of 100 µl.

The results of the preference of NAD(P)H for the protein PA2580 show activity using methyl red with both NADH and NADPH (Figure 4.11 **a**). However, better activity was shown with NADPH (19.72±0.85 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADH (6.68±1.52 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (t-test) was performed showing a significant difference between samples ($p<0.01$), indicating that this protein prefers NADPH for the reduction of substrates.

The NAD(P)H preference results for PA2280 show protein activity using methyl red with either NADH and NADPH (Figure 4.11 **b**) where higher activity was shown with NADPH (10.40±0.26 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADH (3.25±0.82 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (t-test) was performed showing a significant difference between samples ($p<0.01$), indicating that this protein prefers NADPH for the reduction of substrates.

For the protein PA1204, the NAD(P)H preference results show that no activity was obtained using methyl red as substrate (Figure 4.11 **c**). For this reason, a quinone, MEN, was used to perform the same assay. The results show protein activity using MEN with both NADH and NADPH (Figure 4.11 **c**), with better substrate reduction obtained using NADPH (802.8±36.72 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADH (585.1±20.3 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) was performed showing significant difference between MEN+NADPH and MEN+NADH samples ($p<0.01$), indicating that also this protein prefers NADPH.

As was observed for PA1204, no activity was obtained using methyl red as substrate with PA0949 and for this reason MEN was used to perform the same assay. The results show no activity with either substrate and using either NADH and NADPH. This indicates that PA0949 could have been inactive or requires different conditions to enzymatically react.

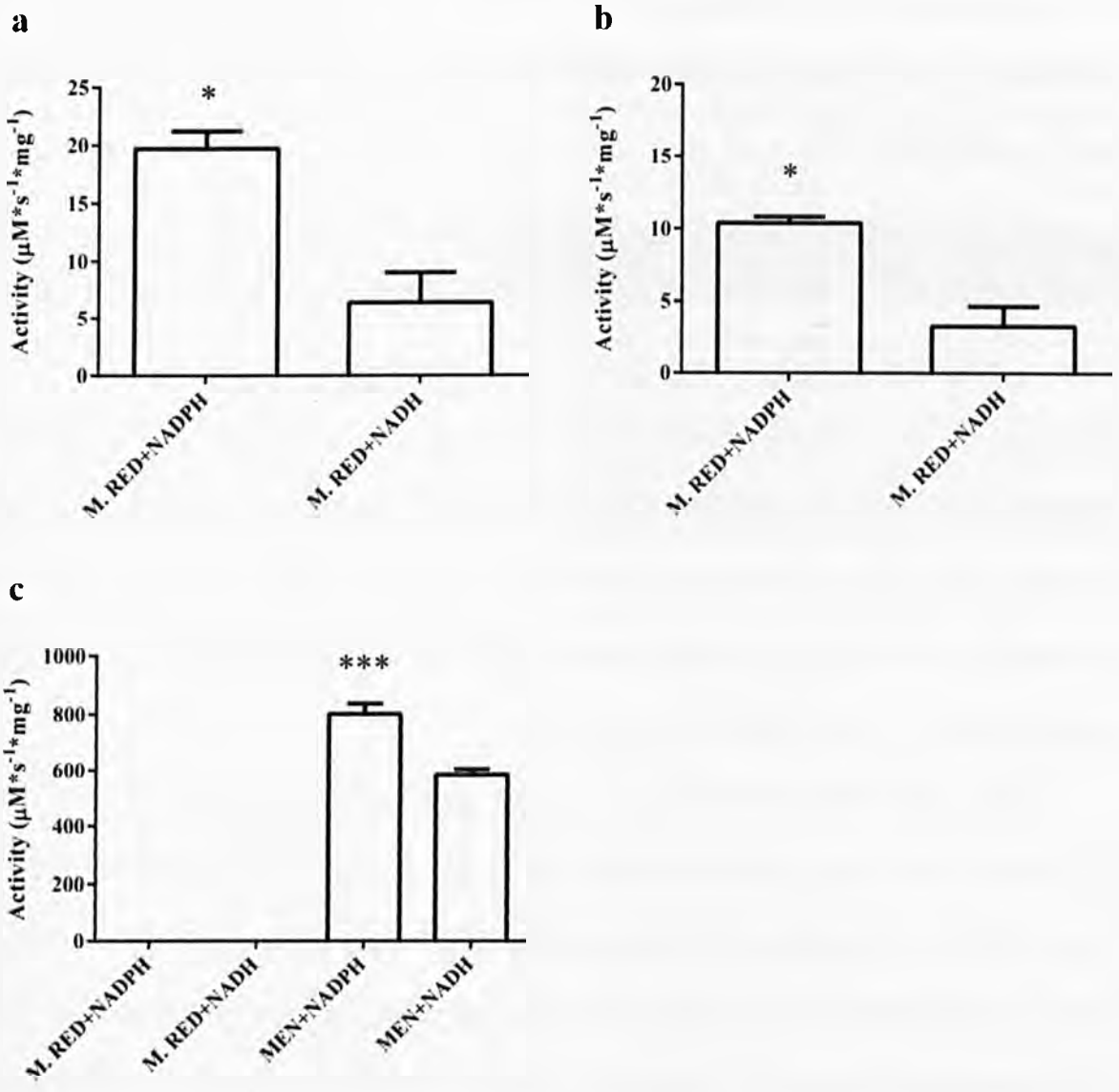


Figure 4.11 Nicotinamide preference of putative *P. aeruginosa* PAO1 azoreductase proteins.

(**a**) PA2580 NAD(P)H preference. Higher reduction was obtained using NADPH (19.72±0.85 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADH (6.38±1.52 $\mu M \cdot s^{-1} \cdot mg^{-1} \pm 1.52$). Statistical analysis (t-test) showed significant differences between samples ($p<0.01$). (**b**) PA2280 NAD(P)H preference. Higher reduction is obtained using NADPH (10.40±0.26 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADH (3.25±0.82 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (t-test) shown significance difference between samples ($p<0.01$). (c) PA1204 NAD(P)H preference. Protein activity was obtained only with MEN, where higher reduction was obtained using NADPH (802.8±36.72 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADH (585.1±20.31 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significance difference between MEN+NADPH and MEN+NADH samples ($p<0.0001$). Reduction rates ($\mu M \cdot s^{-1} \cdot mg^{-1}$) were calculated by fitting the change in OD over 300 seconds. Error bars showing the standard deviation of replicates (n=3). Reactions were prepared using 10 μg of protein, 50 μM of substrate and 500 μM of NAD(P)H in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

To determine the Flavin preference, the same reaction determine the NAD(P)H preference was performed by adding either FMN or FAD, an all reaction combinations are listed in table 4.4.

In the presence of added flavin, the protein PA2580 (Figure 4.12 **a**) shown a higher the reduction rate with FAD (41.80±0.52 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than with FMN (37.14±0.65 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) was performed showing a significant difference between samples containing FAD and samples containing FMN ($p<0.001$), indicating that this protein prefers FAD for the reduction of substrates. This also confirms the TLC results, where FAD was the flavin contained in the protein. Comparison of methyl red reduction with and without flavin showed an increase enzymatic activity of more than one fold when FAD was present in the reaction.

The results for PA2280 (Figure 4.12 **b**) show higher reduction rate with FMN (21.72±0.58 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than FAD (16.03±0.65 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) was performed showing significant difference between samples containing FMN and samples containing FAD ($p<0.0001$), indicating that this protein prefers FMN for the reduction of substrates. Comparison of methyl red reduction with and without flavin shown an increasing enzymatic activity of more than one fold when FMN was present in the reaction.

The protein PA1204 (Figure 4.12 **c**) shows a higher reduction rate with FAD (847.7±18.43 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than FMN (743.9±21.31 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) was performed showing significant difference between samples containing FAD and samples containing FMN ($p<0.01$), indicating that this protein prefers FAD for the reduction of substrates, but appears to work with both flavins.

Table 4.4 Enzymatic reaction components and combinations used to determine the flavin preference.

Eight combinations of reaction components used in order to establish the flavin preference for the enzymatic reaction. Reactions 5, 6, 7 and 8 represent the negative controls. Samples were prepared using 10 µg of protein, 50 µM of substrate, 500 µM of NAD(P)H and either 1 µM of FMN or FAD in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

Enzymatic reaction	protein	Substrate	NADPH	NADH	FMN	FAD
1	X	X	X		X	
2	X	X	X			X
3	X	X		X	X	
4	X	X		X		X
5		X	X		X	
6		X	X			X
7		X		X	X	
8		X		X		X

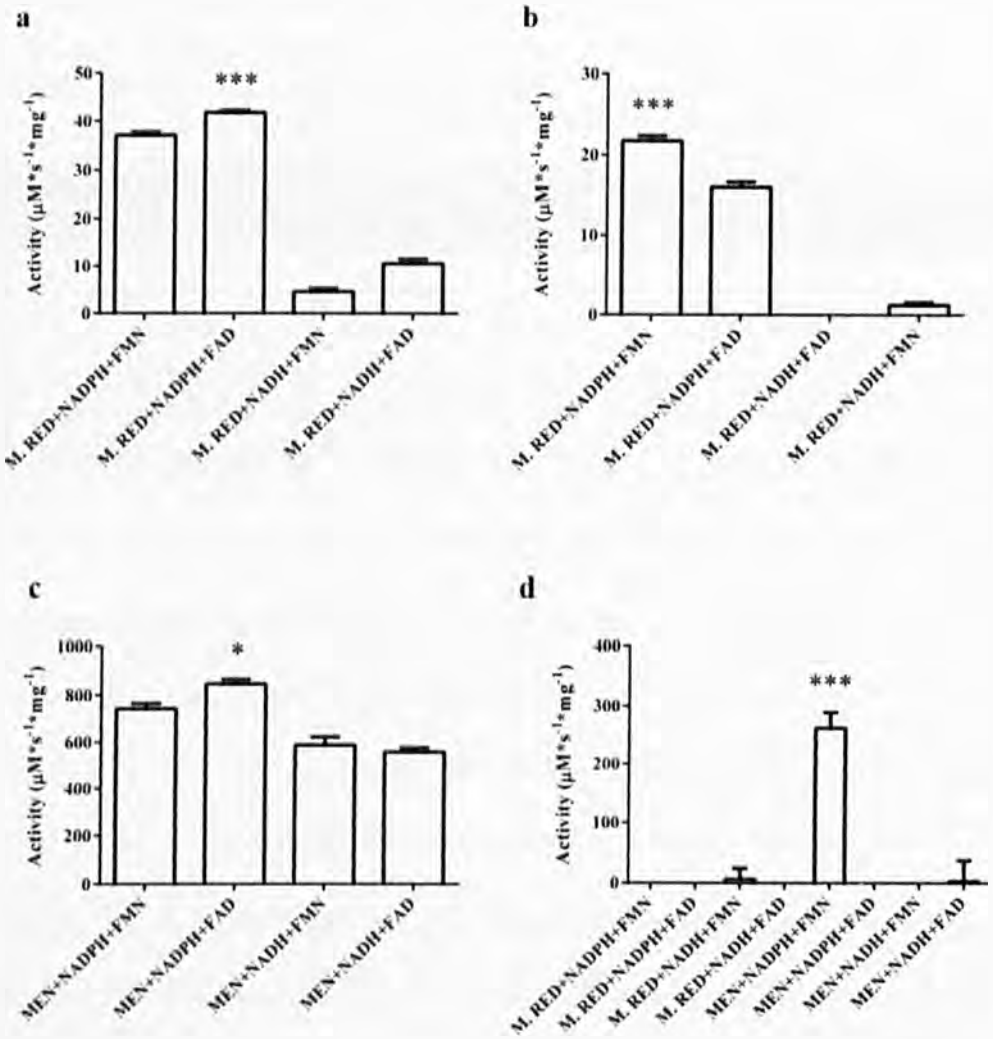


Figure 4.12 Determination of flavin preference of of putative *P. aeruginosa* PAO1 azoreductase proteins.

(**a**) PA2580 flavin preference. Higher reduction was obtained using NADPH+FAD (41.80±0.52 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADPH+FMN (37.14±0.65 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significance difference between M.RED+NADPH+FAD and M.RED+NADPH+FMN samples ($p<0.001$). (**b**) PA2280 flavin preference. Higher reduction was obtained using NADPH+FMN (21.72±0.58 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADPH+FAD (16.03±0.65 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significance difference between M.RED+NADPH+FMN and MEN+NADPH+FAD samples ($p<0.0001$). (**c**) Higher reduction was obtained using NADPH and FAD (847.7±18.43 $\mu M \cdot s^{-1} \cdot mg^{-1}$) than NADPH and FMN (743.9±21.31 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significance difference between MEN+NADPH+FAD and MEN+NADPH+FMN samples ($p<0.01$; significance $p<0.05$). Enzymatic activity was obtained for MEN and only when NADPH and FMN are used (262.6±31.20 $\mu M \cdot s^{-1} \cdot mg^{-1}$). No reduction was found in all other conditions. Statistical analysis (one-way ANOVA) shown significance difference between MEN+NADPH+FMN and all other samples ($p<0.0001$). Reduction rates ($\mu M \cdot s^{-1} \cdot mg^{-1}$) were calculated by fitting the change in OD over 300 seconds. Error bars showing the standard deviation of replicates (n=3). Reactions were prepared using 10 μg of protein, 50 μM of substrate, 500 μM of NAD(P)H and either 1 μM of FMN or FAD in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

Although no activity was found for PA0949 during the NAD(P)H preference assay, it was decided to test this protein using again methyl red and MEN, adding NADPH and NADH and using also FMN and FAD, to determine if this lack of activity was due to insufficient flavin being associated with the protein following the purification process. The Flavin determination assay (Figure 4.12 **d**) shows an enzymatic activity using MEN as substrate only when NADPH and FMN are supplied to the reaction (262.6±31.20 $\mu M \cdot s^{-1} \cdot mg^{-1}$). A small activity level was found for methyl red with NADPH and FMN and for MEN with NADH and FAD. However, these levels of activity are extremely low. These data indicate that PA0949 requires NADPH and FMN at the same time in order to reduce substrates. Statistical analysis (one-way ANOVA) was performed showing significant difference between MEN+NADPH+FMN and all other samples ($p<0.0001$), indicating that this protein prefers FMN and NADPH for the reduction of substrates.

4.2.2.2. Substrate specificity

Previous data showed that azoreductase reduction rate was higher for quinones than for azo substrates. For this reason, the protein concentrations used were from 0.15 to 20 μg for azo compounds and 0.04 to 5 μg for quinones. Also in this case, absorbance was measured every 15 second for 30 minutes obtaining nine reduction profiles for each compound tested (Figure 4.13) and rates were calculated.

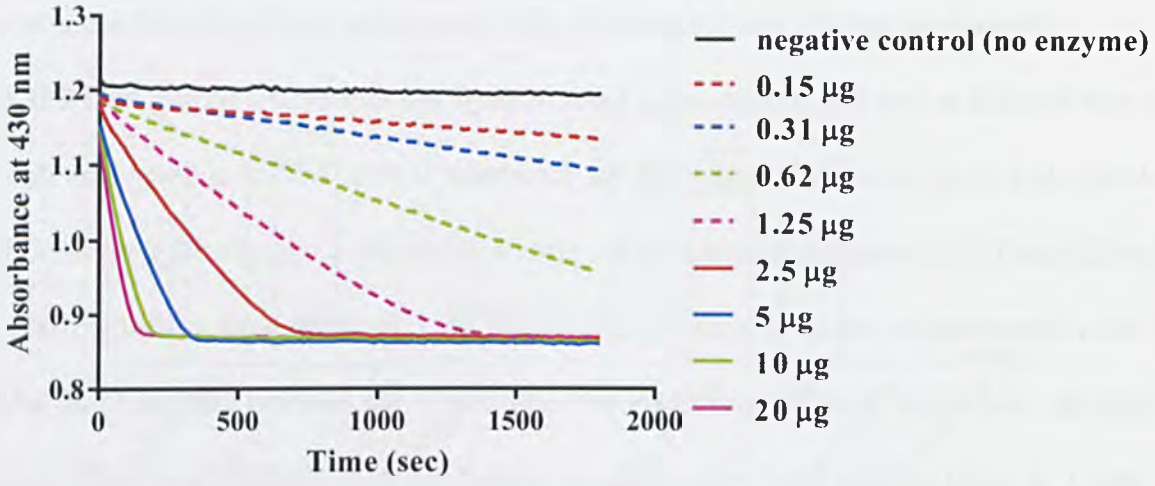


Figure 4.13 Effect of protein concentration on rate of reduction of methyl red by PA2580.

Graph showing methyl red absorbance measurements during its reduction by PA2580 using NADPH and FAD. Eight protein concentrations (20-0.15 µg) and negative control (no protein, black line) were used and absorbance was measured every 15 seconds for 30 minutes. Decreasing of absorbance levels represent the reduction of Methyl red while the reactions proceed. No absorbance changes were observed for negative control, indicating that no reduction of substrate was occurring. Reactions were prepared in replicates (n=3) and using 10 µg of protein, 50 µM of substrate, 500 µM of NADPH and 1 µM of FAD in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

4.2.2.2.1. PA2580

The enzymatic activity of PA2580 with the azo compounds (Figure 4.14 **a**) show that this enzyme reduces methyl red (37.9±1.93 $\mu M \cdot s^{-1} \cdot mg^{-1}$), sudan I (27.23±2.28 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and balsalazide (12.46±3.45 $\mu M \cdot s^{-1} \cdot mg^{-1}$), whereas no activity was found with any other azo compounds. Statistical analysis (one-way ANOVA) showed significant difference between methyl red and the other two reduced substrates ($p<0.0001$), indicating methyl red as best azo substrate for PA2580.

Regarding the enzymatic activity using quinones as substrates, the results show that PA2580 was able to reduce almost all quinones tested (Figure 4.14 **b**), but at different levels across a wide range. The highest reduction rate was observed with MEN (909.8±51.69 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and PLU (894.4±30.25 $\mu M \cdot s^{-1} \cdot mg^{-1}$) for which no significant difference has been found (one-way ANOVA analysis), indicating that both can be considered the preferred substrate for this enzyme. This is also confirmed by the significant difference between these two substrates and all the others ($p<0.0001$). This enzyme also showed high reduction rate with HNQ (639.4±51.87 $\mu M \cdot s^{-1} \cdot mg^{-1}$), but less compared with MEN and PLU.

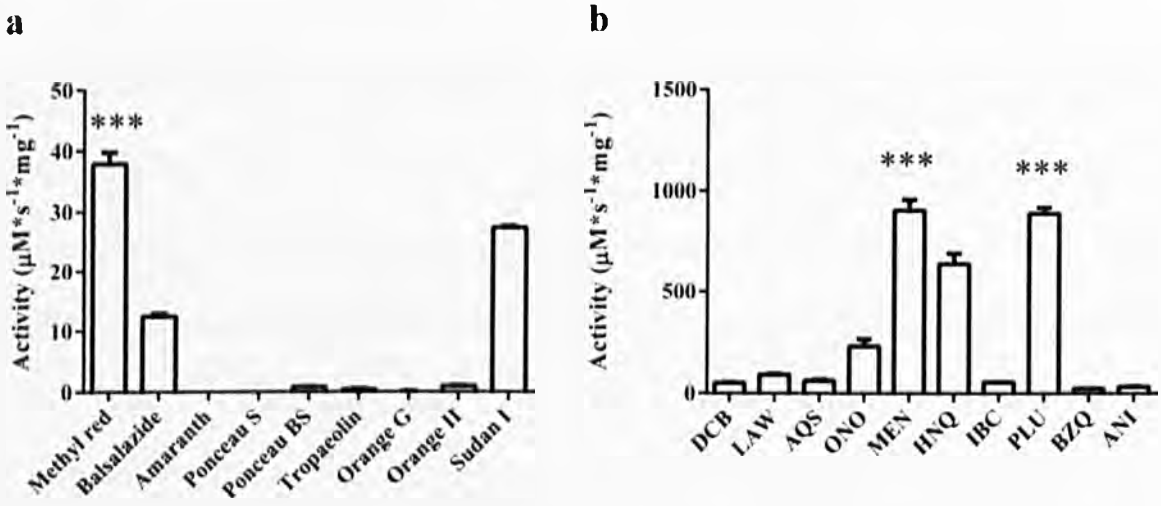


Figure 4.14 PA2580 enzymatic profile specificity.
(**a**) PA2580 reduction rates of azo compounds. Enzymatic activity obtained with methyl red (37.9±1.93 $\mu M \cdot s^{-1} \cdot mg^{-1}$), sudan I (27.23±2.28 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and balsalazide (12.46±3.45 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significance difference between reduction rate of methyl red and the other two substrates ($p<0.0001$). (**b**) PA2580 reduction rates of quinones. Enzymatic activity obtained with MEN (909.8±51.69 $\mu M \cdot s^{-1} \cdot mg^{-1}$), PLU (894.4±30.25 $\mu M \cdot s^{-1} \cdot mg^{-1}$), HNQ (639.4±51.87 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ONO (231.6±34.42 $\mu M \cdot s^{-1} \cdot mg^{-1}$), LAW (90.86±4.46 $\mu M \cdot s^{-1} \cdot mg^{-1}$), AQS (62.49±5.49 $\mu M \cdot s^{-1} \cdot mg^{-1}$), IBC (56.05±1.15 $\mu M \cdot s^{-1} \cdot mg^{-1}$), DCB (50.24±4.26 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ANI (32.76±2.31 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and BZQ (24.15±6.91 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significance difference between the two conditions MEN and PLU, and all the other substrates ($p<0.0001$). Reduction rates ($\mu M \cdot s^{-1} \cdot mg^{-1}$) were calculated by fitting the change in OD over 300 seconds. Error bars showing the standard deviation of replicates (n=3). Reactions were prepared using between 0 and 20 (with a azo compounds) or 0 and 5 (with quinones) μg of protein, 50 μM of substrate, 500 μM of NADPH and 1 μM of FMN in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

4.2.2.2.2. PA2280

The PA2280 enzymatic activity with azo compounds (Figure 4.15 **a**) shows that this enzyme reduces only a few of the substrates tested including sudan I (134.1±2.08 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ponceau S (30.65±5.96 $\mu M \cdot s^{-1} \cdot mg^{-1}$), balsalazide (7.41±1.47 $\mu M \cdot s^{-1} \cdot mg^{-1}$), amaranth (5.81±1.35 $\mu M \cdot s^{-1} \cdot mg^{-1}$), methyl red (4.99±5.36 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and ponceau BS (1.41±0.07 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) has shown that the reduction of sudan I is significant different from the others ($p<0.0001$), indicating that this is the best azo substrate for PA2280.

A different situation has been observed with the quinones tested. PA2280 was able to reduce all quinones used, although with different activities (Figure 4.15 **b**). A high reduction rate was shown with ANI (821.9±32.36 $\mu M \cdot s^{-1} \cdot mg^{-1}$), relatively medium activity with HNQ (396.3±10.58 $\mu M \cdot s^{-1} \cdot mg^{-1}$), LAW (340.8±17.47 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and PLU (237.4±6.97 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and low activity with MEN (127.1±8.39 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ONO (87.68±7.26 $\mu M \cdot s^{-1} \cdot mg^{-1}$), DCB (71.32±6.53 $\mu M \cdot s^{-1} \cdot mg^{-1}$), AQS (51.03±3.94 $\mu M \cdot s^{-1} \cdot mg^{-1}$), IBC (49.69±1.90 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and BZQ (30.55±1.23 $\mu M \cdot s^{-1} \cdot mg^{-1}$). ANI is the best substrate for PA2280 and this was also supported by statistical analysis (one-way ANOVA) showing significant difference between the reduction of this substrate and all others ($p<0.0001$).

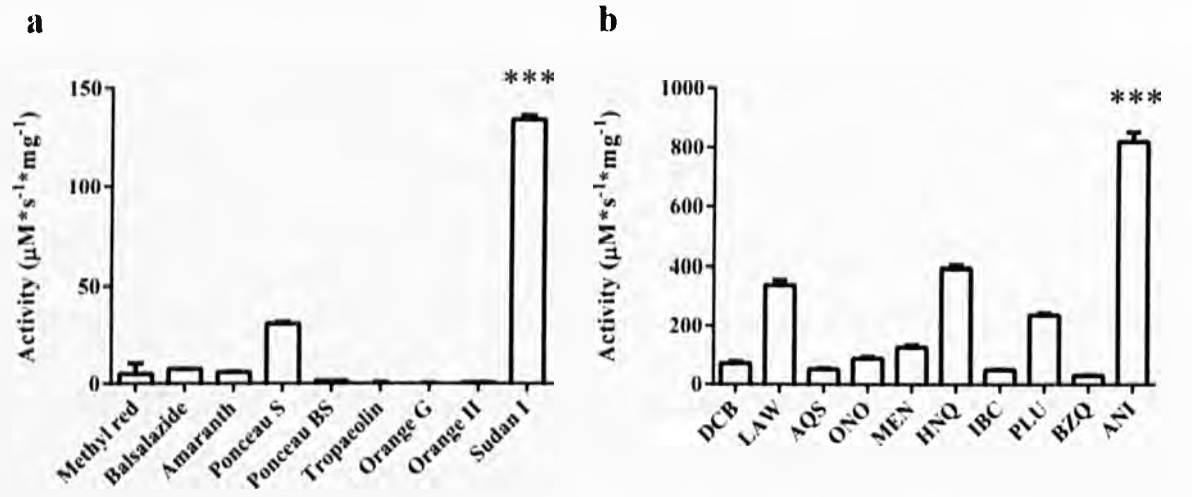


Figure 4.15 PA2280 enzymatic profile specificity.
(**a**) PA2280 reduction rates of azo compounds. Enzymatic activity obtained with sudan I (134.1±2.08 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ponceau S (30.65±5.96 $\mu M \cdot s^{-1} \cdot mg^{-1}$), balsalazide (7.41±1.47 $\mu M \cdot s^{-1} \cdot mg^{-1}$), amaranth (5.81±1.35 $\mu M^* s^{-1} * mg^{-1}$), methyl red (4.99±5.36 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and ponceau BS (1.41±0.07 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) show significant difference between Sudan I and all other conditions ($p<0.0001$). (**b**) PA2280 reduction rates of quinones. Enzymatic activity obtained with ANI (821.9±32.36 μM·s-1·mg-1), HNQ (396.3±10.58 μM·s-1·mg-1), LAW (340.8±17.47 μM·s-1·mg-1) and PLU (237.4±6.97 μM·s-1·mg-1) and low activity with MEN (127.1±8.39 μM·s-1·mg-1), ONO (87.68±7.26 μM·s-1·mg-1), DCB (71.32±6.53 μM·s-1·mg-1), AQS (51.03±3.94 μM·s-1·mg-1), IBC (49.69±1.90 μM·s-1·mg-1) and BZQ (30.55±1.23 μM·s-1·mg-1). Reduction rates ($\mu M \cdot s^{-1} \cdot mg^{-1}$) were calculated by fitting the change in OD over 300 seconds. Error bars showing the standard deviation of replicates (n=3). Reactions were prepared using between 0 and 20 (with a azo compounds) or 0 and 5 (with quinones) μg of protein, 50 μM of substrate, 500 μM of NADPH and 1 μM of FMN in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

4.2.2.2.3. PA1204

The substrate specificity analysis for the protein PA1204 showed a poor reduction of only few azo compound (Figure 4.16 **a**). In particular, reduction of orange G (8.49±2.93 $\mu M \cdot s^{-1} \cdot mg^{-1}$), balsalazide (4.6±0.63$\mu M \cdot s^{-1} \cdot mg^{-1}$) and amaranth (0.4±0.07 $\mu M \cdot s^{-1} \cdot mg^{-1}$) only were observed, whereas no reduction was obtained with all other azo substrates. The graph shows that orange G was the best substrate for this enzyme. This was confirmed with statistical analysis (one-way ANOVA), which shown a significant difference between orange G and all other azo substrates ($p<0.01$).

Regarding the substrate specificity reduction profiles with quinones (Figure 4.16 **b**), PA1204 showed a high reduction of ONO (6640±1563 $\mu M \cdot s^{-1} \cdot mg^{-1}$), a relatively moderate reduction of IBC (3404±809.08 $\mu M \cdot s^{-1} \cdot mg^{-1}$), MEN (2860±29.60 $\mu M \cdot s^{-1} \cdot mg^{-1}$), PLU (2762±83.21 $\mu M \cdot s^{-1} \cdot mg^{-1}$), HNQ (1997±217.08 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and ANI (1454±58.04 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and a low reduction of AQS (51.03±3.44 $\mu M \cdot s^{-1} \cdot mg^{-1}$), DCB (49.69±6.02 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and BZQ (30.55±1.39 $\mu M \cdot s^{-1} \cdot mg^{-1}$). ONO was the best quinone substrate for this enzyme, also confirmed with statistical analysis ($p<0.0001$)

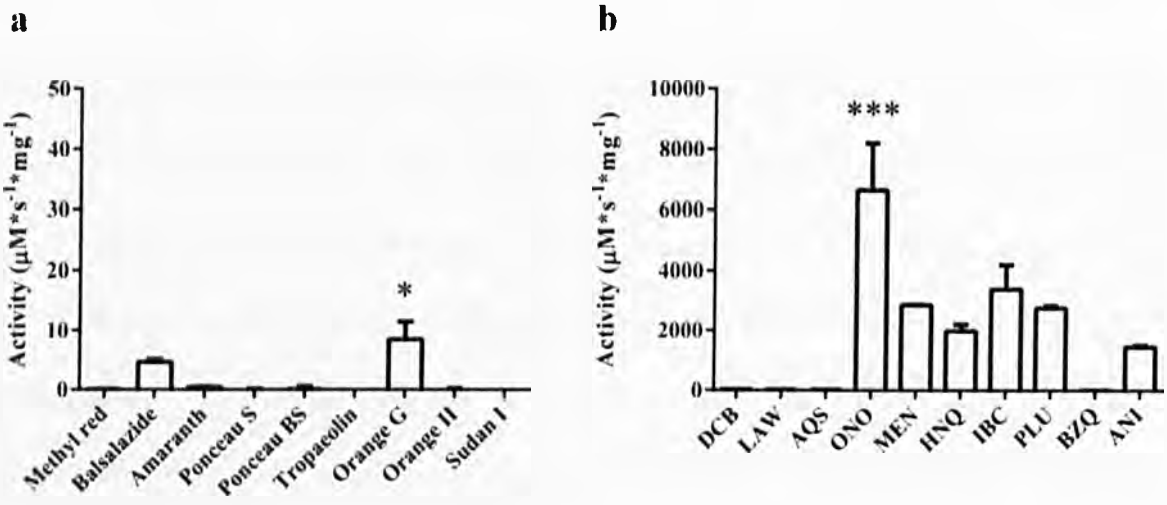


Figure 4.16 PA1204 enzymatic profile specificity.
(**a**) PA1204 reduction rates of azo compounds. Enzymatic activity obtained with Orange G (8.49±2.93 $\mu M \cdot s^{-1} \cdot mg^{-1}$), balsalazide (4.6±0.63 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and amaranth (0.4±0.07 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significant difference between Orange G and all other azo substrates ($p<0.01$). (**b**) PA1204 reduction rates of quinones. Enzymatic activity obtained with ONO (6640±1563 μM·s-1·mg-1), IBC (3404±809.08 μM·s-1·mg-1), MEN (2860±29.60 μM·s-1·mg-1), PLU (2762±83.21 μM·s-1·mg-1), HNQ (1997±217.08 μM·s-1·mg-1), ANI (1454±58.04 μM·s-1·mg-1), AQS (51.03±3.44 μM·s-1·mg-1), DCB (49.69±6.02 μM·s-1·mg-1) and BZQ (30.55±1.39 μM·s-1·mg-1). Statistical analysis (one-way ANOVA) shown significant difference between ONO and all other azo substrates ($p<0.0001$). Reduction rates ($\mu M \cdot s^{-1} \cdot mg^{-1}$) were calculated by fitting the change in OD over 300 seconds. Error bars showing the standard deviation of replicates (n=3). Reactions were prepared using between 0 and 20 (with azo compounds) or 0 and 5 (with quinones) μg of protein, 50 μM of substrate, 500 μM of NADPH and 1 μM of FAD in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

4.2.2.2.4. PA0949

The enzymatic activity of PA0949 with azo substrates was shown to be really poor (Figure 4.17 **a**). Enzymatic activity was observed with some substrates, however because of high replicate variability (sudan I and orange G) or reduction rates of less than 1 $\mu M \cdot s^{-1} \cdot mg^{-1}$ (ponceau S, ponceau BS, methyl red and tropaeolin), it cannot be considered a proper substrate reduction. This was also confirmed by statistical analysis (one-way ANOVA) which hasn't shown any significant difference between any substrate. For this reason, it can be considered that PA0949 does not reduce the azo substrates tested.

Although poor activity was observed with azo compounds, PA0949 has been shown to be able to reduce quinones (figure 4.17 **b**). Reduction by PA0949 of MEN (363.3 ± 37.36 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ANI ($291.3 \pm 26.30$ $\mu M \cdot s^{-1} \cdot mg^{-1}$), PLU ($229.1 \pm 28.50$ $\mu M \cdot s^{-1} \cdot mg^{-1}$) was the most marked whilst there was clearly detectable reduction of HNQ (95.13 ± 1.01 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and IBC ($56.33 \pm 0.51$ $\mu M \cdot s^{-1} \cdot mg^{-1}$) and a very low, but measurable, reduction of ONO (11.92 ± 2.47 $\mu M \cdot s^{-1} \cdot mg^{-1}$), LAW ($5.6 \pm 1.34$ $\mu M \cdot s^{-1} \cdot mg^{-1}$) and AQS ($2.86 \pm 2.57$ $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis was performed (one-way ANOVA) showing a significant difference between the reduction of MEN and all other substrates ($p<0.01$), indicating that MEN was the preferred substrate for PA0949.

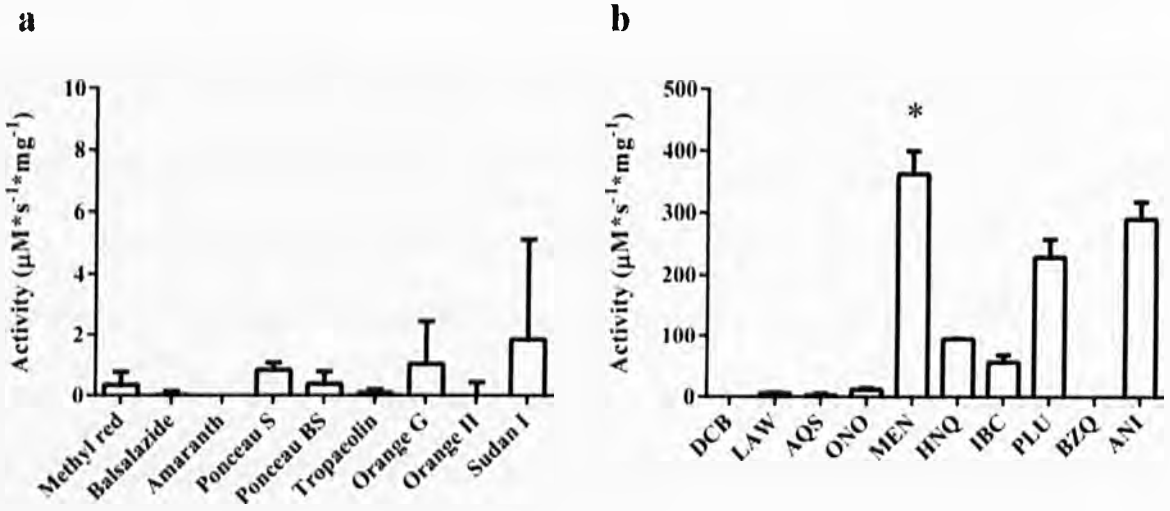


Figure 4.17 PA0949 enzymatic profile specificity with azo compounds.
(**a**) PA0949 reduction rates of azo compounds. No considerable activity (< 1 $\mu M \cdot s^{-1} \cdot mg^{-1}$) or variability between replicated to high (errors between replicates too high) was obtained with all azo substrates tested. (**b**) PA0949 reduction rates of quinones. Enzymatic activity obtained with MEN (363.3±37.36 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ANI (291.3±26.30 $\mu M \cdot s^{-1} \cdot mg^{-1}$), PLU (229.1±28.50 $\mu M \cdot s^{-1} \cdot mg^{-1}$), HNQ (95.13±1.01 $\mu M \cdot s^{-1} \cdot mg^{-1}$), IBC (56.33±0.51 $\mu M \cdot s^{-1} \cdot mg^{-1}$), ONO (11.92±2.47 $\mu M \cdot s^{-1} \cdot mg^{-1}$), LAW (5.6±1.34 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and AQS (2.86±2.57 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Statistical analysis (one-way ANOVA) shown significant difference between the reduction of MEN and all other substrates ($p<0.01$). Reduction rates ($\mu M \cdot s^{-1} \cdot mg^{-1}$) were calculated by fitting the change in OD over 300 seconds. Error bars showing the standard deviation of replicates (n=3). Reactions were prepared using between 0 and 20 (with azo compounds) or 0 and 5 (with quinones) μg of protein, 50 μM of substrate, 500 μM of NADPH and 1 μM FMN in 20 mM TrisHCl 100 mM NaCl pH 8 buffer.

The enzymatic characterization of PA2580, PA2280, PA1204 and PA0949 shown that these proteins varied in their preferred reductant, cofactor and substrate. For PA2580, PA2280 and PA1204 the preferred reductant used during the reduction of substrates was shown to be NADPH, although activity was also observed with NADH. For PA0949, the enzymatic activity was detected only in presence of NADPH and only when exogenous FMN was added to the reaction mixture (Table 4.5).

Enzymatic analysis in the presence of FMN or FAD shows that PA2580 and PA1204 have higher enzymatic activity when FAD, whereas for PA2280 and PA0949, FMN was observed to be the preferred flavin. Comparison of reactions with and without exogenous flavin shows that PA2580, PA2280 and PA1204 exhibit higher activity when exogenous flavin is added, and PA0949 was observed to be active only in presence of exogenous flavin (Table 4.6).

Regarding the substrate specificity of these enzymes, higher enzymatic activity was always observed in presence of quinones. In particular, two aromatic ring naphthoquinones shown to be the preferred substrate used by these enzymes (Table 4.7).

Table 4.5 Comparison of enzymatic rates of putative azoreductase proteins with NADH and NADPH.
Table comparing the reduction rates using NADH or NADPH as electron donor during the enzymatic reduction by PA2580, PA2280, PA1204 and PA0949. All proteins show higher rate in the presence of NADPH.

Protein	Enzymtic rate ($\mu M \cdot s^{-1} \cdot mg^{-1}$) NADH	NADPH	Substrate
PA2580	6.38±1.52	19.72±0.85	Methyl red
PA2280	3.25±0.82	10.4±0.26	Methyl red
PA1204	585.1±20.31	802.8±36.72	MEN
PA0949 (+FMN)	-	262.6±31.20	MEN

Table 4.6 Comparison of enzymatic rates of putative azoreductase proteins with FAD, FMN and without flavin.
Table showing the reduction rates comparison using no flavin, FAD and FMN during the enzymatic reduction by PA2580, PA2280, PA1204 and PA0949. PA2580 and PA1204 show a FAD preference and PA2280 shows a FMN preference. PA0949 shows activity only in presence of FMN.

Protein	Reductant	Enzymatic activity ($\mu M \cdot s^{-1} \cdot mg^{-1}$) no flavin	+ FAD	+ FMN	Substrate
PA2580	NADPH	19.72±0.85	41.8±0.52	37.14±0.65	Methyl red
PA2280	NADPH	10.4±0.26	16.03±0.65	21.72±0.58	Methyl red
PA1204	NADPH	802.8±36.72	847.7±18.43	743.9±21.31	MEN
PA0949	NADPH	-	-	262.6±31.20	MEN

Table 4.7 Five best reduced quinones the by *P. aeruginosa* PAO1 azo and quinone oxidoreductases PA2580, PA2280, PA1204 and PA0949.
Table showing the five best reduced quinones by PA2580, PA2280, PA1204 and PA0949. Red = naphtoquinone; Blue = quinonemine; (*) = one aromatic ring; (**) = two aromatic rings.

Protein	Highest reduced quinone substrates 1st	2nd	3rd	4th	5th
PA2580	MEN (**)	PLU (**)	HNQ (**)	ONO (**)	LAW (**)
PA2280	ANI (*)	HNQ (**)	LAW (**)	PLU (**)	MEN (**)
PA1204	ONO (**)	IBC (**)	MEN (**)	PLU (**)	HNQ (**)
PA0949	MEN (**)	ANI (*)	PLU (**)	HNQ (**)	IBC (**)

4.3. Discussion

The aims described in section 1.6 have been met as follow:

Biochemical and enzymatic analysis of putative *P. aeruginosa* PAO1 azoreductase recombinant proteins

a. Determination of prosthetic group, cofactor and enzymatic reaction conditions for the putative *P. aeruginosa* PAO1 recombinant proteins PA2580, PA2280, PA1204 and PA0949.
b. Determinations of the specific enzymatic activity of these protein with a range of azo compounds and quinone.

Biochemical and enzymatic analysis of four new putative azoreductases proteins have been performed and described here.

The results shown in this chapter indicate that the four proteins PA2580, PA2280, PA1204 and PA0949 have different reductant, cofactor and substrate preference. For PA2580, PA2280 and PA1204 the preferred reductant used during the reduction of substrates was shown to be NADPH, although activity was also observed with NADH. This is consistent with what has been previously found for PA1204 (Agarwal et al., 2006). The use of both reductants, although with different activity level, has been already described for the azoreductases paAzoR1-3 in *P. aeruginosa* PAO1 (Ryan et al., 2010a, Wang et al., 2007). This feature is almost unique amongst the reported bacterial azoreductases. Azoreductase activity from *Proteus vulgaris*, *Bacillus* sp. strain OY1-2 and *S. aereus* has been reported to be NADPH-dependent (Suzuki et al., 2001, Chen et al., 2005a, Roxon et al., 1967), and azoreductases from *E. coli*, *E. faecalis*, *R. sphaeroides* AS1.1737, *E. agglomerans* and *Bacillus* sp. strain SF have been reported to use only NADH (Moutaouakkil et al., 2003, Bin et al., 2004, Nakanishi et al., 2001, Chen et al.,

2004, Maier et al., 2004). Regarding PA0949, enzymatic activity has been observed only in the presence of NADPH, indicating that this enzyme acts differently, in terms of reductant dependence, from the other reported *P. aeruginosa* PAO1 azoreductases, but similarly to the reported bacterial azoreductases.

The protein spectral analyses show the presence of defined flavin peaks for the proteins PA2580, PA1204 and less defined for PA2280 and PA0949. Similarly, TLC analysis showed the presence of FAD for PA2580 and PA1204. These data confirm what has been found for the *E. coli* MdaB, which shows to use FAD as a cofactor during enzymatic reduction of quinone substrates (Hayashi et al., 1996). In the case of PA2280 and PA0949 no flavin was detected. However, when flavin was added during the enzymatic assays, the activity of PA2280 and PA0949 increased. These data suggest that the flavin molecules could become dissociated from the recombinant proteins or not sufficiently incorporated during the protein production process. This is a frequent problem during the expression of these proteins. For example, it was observed for the crystallization of PA0949 and PA1204, which was gained only by adding FMN to preformed crystals (Agarwal et al., 2006, Gorman and Shapiro, 2005).

Enzymatic analysis in the presence of FMN or FAD confirmed the findings obtained for PA2580 and PA1204, showing higher enzymatic activity when FAD is used, whereas for PA2280 and PA0949, FMN was observed to be the preferred flavin during the enzymatic reactions. Several studies reported that the addition of exogenous flavin molecules to a growing bacterial culture or to enzymatic reaction mixtures enhances the reduction rate (Rafii et al., 1990, Chung et al., 1978, Maier et al., 2004, Mallett et al., 1982). However, no activity changes have been observed for the three azoreductases (paAzoR1-3) previously described in *P. aeruginosa* PAO1.

In the study described in this chapter, the enzymatic activity with and without adding exogenous flavin molecules shows that PA2580, PA2280 and PA1204 exhibit

higher activity when exogenous flavin is added to the reaction mixture. This effect was even more important for PA0949, for which enzymatic activity was observed only in presence of exogenous flavin.

Regarding the enzymatic activity with azo compound and quinones, the results described in this chapter indicate that the four proteins analysed exhibited a very diverse substrate specificity. With the azo substrates PA2280 was shown to be the most active protein (134.1±2.08 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and PA1204 is least active (8.49±2.93 $\mu M \cdot s^{-1} \cdot mg^{-1}$), whereas no activity has been shown with PA0949. With the quinones, PA1204 showed the highest activity (6640±1563 $\mu M \cdot s^{-1} \cdot mg^{-1}$) and PA0949 the lowest (363.3±37.36 $\mu M \cdot s^{-1} \cdot mg^{-1}$). Considering the best quinones reduced for each enzyme, it has been observed that naphthoquinones and, in some cases, quinonimine are the substrates preferred by these enzymes. Interestingly, the majority of the quinones reduced have two aromatic rings, except in the case of PA2280, which shows the highest reduction with ANI, a single aromatic ring quinonemine. This preference could be explained by the size of the active site. Previous studies showed that paAzoR2 and paAzoR3 can reduce larger substrates than paAzoR1. This was explained by comparing the structure of paAzoR1 and homology models of paAzoR2 and paAzoR3 and showing a smaller active site for paAzoR1 protein compared with the other two (Ryan et al., 2010a, Gonçalves et al., 2013, Ito et al., 2008). Following this hypothesis, PA2580, PA1204 and PA0949 could be explained to have larger active sites then PA2280, and consequently able to bind larger substrates such as the naphthoquinones. This is also supported by experimental enzymatic data showing that the enzymes paAzoR2 and paAzoR3 show higher reduction rates than paAzoR1 with naphtoquinones (Ryan et al., 2014).

For all four proteins described in this chapter it has been observed that the activity with quinones was higher than the azo compounds. In fact, considering the highest rates, the reduction of quinones by PA2580, PA2280 and PA1204 is 24, 6 and 782 fold higher

respectively than the reduction of azo compounds and PA0949 does not show any azo compound reduction at all. This suggests that these enzymes are better suited for carrying out quinone rather than azo reduction, confirming previous data proposing that azoreductases from *P. aeruginosa* PAO1 are able to better reduce quinone substrates, and should be considered quinone oxidoreductases (Wang et al., 2007, Ryan et al., 2010a, Ryan et al., 2014). However, it is not clear what their physiological role is. For this reason, experiments using azoreductase gene transposon mutants were carried out to address this question which is described in the next chapter.

Summary

Biochemical and enzymatic analysis has been performed for the recombinant proteins PA0949, PA1204, PA2280 and PA2580 showing that:

1. Flavin molecules were detected in PA2580 and PA1204, but not in PA2280 and PA0949. Addition of exogenous FAD for PA2580 and PA1204 and FMN for PA2280 and PA0949 enhanced their enzymatic activity.
2. NADPH has been shown to be the preferred electron donor during enzymatic reactions.
3. PA2580, PA2280 and PA1204 are able to reduce both azo- and quinone- substrates with higher reduction rate with quinones. PA0949 reduced only quinones.
4. Naphthoquinones, which are two aromatic rings molecules, are shown to be the preferred substrates for all four enzymes tested.

These finding prove that PA2580, PA2280 and PA1204 are flavodoxin enzymes that can be classified as azo reductases and more precisely quinone oxidoreductases. Due to the absence of activity with azo compounds, PA0949 can be described as quinone oxidoreductase only. It has also been proved that these enzymes use NADPH during the reduction of substrates, and they generally prefer two aromatic ring molecules such as

naphthoquinones. The results presented here pave the way for identifying the physiological role of these enzymes and the following chapter describes a genetic approach to understand the role of the genes which encode for these enzymes.

Chapter 5. Characterization of *P. aeruginosa* PAO1 azoreductase genes: effect on bacterial physiology

5.1. Introduction

P. aeruginosa is a Gram-negative bacterium able to colonize a wide range of environmental habitats, and it is a primary agent of opportunistic infection in humans, causing both acute and chronic infections (Driscoll et al., 2007, Ringen and Drake, 1952, Green et al., 1974a, Pellett et al., 1983a).

Many *P. aeruginosa* traits that contribute to human virulence can be correlated to a particular function relevant to its environmental lifestyle and it has been hypothesized that the virulence factors used by *P. aeruginosa* to infect humans are the results of defence mechanisms developed by this bacterium (Hilbi et al., 2007). One of the most important defence mechanism utilized by *P. aeruginosa* is the formation of biofilms, multicellular communities surrounded by a self-produced polysaccharide matrix.

Biofilms are intrinsically resistant to stress conditions and provide protection from both toxic and antibacterial compounds in the environment, as well as predation (Matz and Kjelleberg, 2005). Chronic infections involve the formation of biofilms, which in the context of human infection, protect against assault by the host immune system and provide resistance to antibiotics (Donlan and Costerton, 2002, Ryder et al., 2007). Another important environmental adaptation which has been correlated with virulence in *P. aeruginosa* is an increase of swarming activity. This feature leads to a greater antibiotic resistance (Overhage et al., 2008, Lai et al., 2009).

P. aeruginosa infections are also difficult to treat because of their low susceptibility to antimicrobial agents and the emergence of antibiotic resistance during therapy (Carmeli et al., 1999, Garner et al., 1988). The increased level of drug resistance is a result of the emergence of new resistance in a specific organism after exposure to antimicrobials as

well as of patient-to-patient spread of resistant organisms (Carmeli et al., 1999, Fridkin and Gaynes, 1999). Accumulation of resistance and cross-resistance between agents may result in the formation of MDR *P. aeruginosa* strains.

Azoreductases have been described to cleava the azo bond (-N=N-) found in azo dyes and azo pro-drugs via a ping pong bi bi mechanism (Nakanishi et al., 2001). These enzymes are found in many organisms and bacteria such as *P. aeruginosa* and enterobacteria, including *E. coli*, *E. faecalis* and *Lactobacillus* sp. and it is unlikely that their primary function is the reduction of these synthetic substances (Wang et al., 2007).

Although azoreductases have been originally identified and characterized for their ability to reduce azo compounds, subsequent studies have shown that they are able to reduce quinones (Ryan et al., 2014). Additionaly it has been shown in the work presented in this thesis (chapter 4) that azoreductases from *P. aeruginosa* have higher rates of reduction against quinones than azo substrates, suggesting that their principle biological role is actually the detoxification of quinones (Ryan et al., 2011, Ryan et al., 2014, Ryan et al., 2010a, Liu et al., 2009b, Liu et al., 2009a).

Although azoreductases have been characterised in many bacterial species their physiological role in *P. aeruginosa* remains unclear. However, some studies on these enzymes and their genes have suggested a new hypothesis on their physiological role. For example, *paazor1* gene has been shown to be critical for bacterial colonisation during chronic lung infection of mouse model and important for bacterial growth on a number of different carbon and nitrogen sources (Wiehlmann and Tummler, 2007, Rakhimova et al., 2008). In *H. pylori* the homologue of azoreductase named MdaB, which shares 55% identity to the equivalent *pa2580* gene in *P. aeruginosa* and same enzyme activities, has been shown to play a role in the host infection process (Wang and Maier, 2004). Similar results were found in *Francisella novicida*, where MdaB was shown to be involved in lung and liver infections in mouse (Kraemer et al., 2009). Microarray studies on the Virulence

and quorum sensing Regulator (VqsR, *pa2591*) in *P. aeruginosa* has been shown to regulate both *paazor1* and *paazor2* (Juhas et al., 2005, Li, 2007). Another two genes encoding for azoreductase and quinone oxidoreductase enzymes (chapter 4), *pa0949* and *pa2280*, have been shown to be regulated by PhoQ (*pa1180*) another important regulator involved with virulence (Gooderham et al., 2009b). A study on *P. aeruginosa* antibiotic resistance shown that the *pa2580* knock out strain exhibits an increase of sensitivity to antibiotic treatments (Chen et al., 2010b). Over expression of the *E. coli* azoreductase homologues AzoR and MdaB (*E. coli* cells that contain plasmids carrying azoreductase genes) leads to increases in MICs with the aminoglycoside antibiotics (streptomycin and kanamycin) in comparison with the wild type (Ling et al., 2012). Microarray data shown that *P. aeruginosa* BexR regulates the transcription of the *pa1202* operon which includes *pa1204* gene (*pa1202-pa1205*) and BexR mutants show a 2-fold increase in sensitivity to the fluoroquinolone antibiotic ciprofloxacin, which is the commonly used for treatments of *P. aeruginosa* in CF patients (Breidenstein et al., 2008, Gibson et al., 2003, Turner et al., 2009). All of these data suggest that azoreductases may play an as yet unidentified but crucial role in bacterial host infection and/or antibiotic resistance in *P. aeruginosa*.

This chapter describes the effects of mutating azoreductase genes on some *P. aeruginosa* PAO1 physiological behaviours such as bacterial swarming, biofilm production and antibiotic resistance. To do this, single gene transposon mutants for the genes *paazor1* (*pa0785*), *paazor2* (*pa1962*), *paazor3* (*pa3223*), *pa2580*, *pa2280*, *pa1204*, *pa0949* and *pa4975* were used.

5.2. Results

To study the physiological role of azoreductase genes in *P. aeruginosa* PAO1 in relation to physiological processes including motility, biofilm production and antibiotic resistance, single gene transposon mutants for each of the azoreductase genes were used.

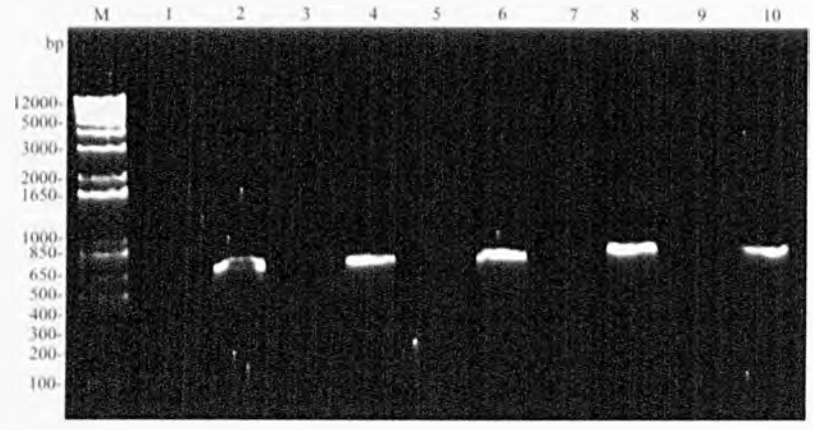
The experiments described here show the comparison between *P. aeruginosa* PAO1 wild type and single gene transposon mutants in relation to growth, motility, biofilm production and antibiotic resistance. These comparisons were made to pin down any possible correlation between genes of interest and physiological behaviour.

5.2.1. Characterization of *P. aeruginosa* PAO1 single gene transposon mutants

P. aeruginosa PAO1 wild type and single gene transposon mutant strains were received from a commercial source in stab cultures on LB agar. It was therefore important to characterise them. To do this, each azoreductase gene, from *P. aeruginosa* PAO1 wild type and mutant strains, was amplified via PCR using gene specific primers. Considering that transposon mutants were generated inserting a DNA sequence of 4.83 or 6.16 Kbp into the gene coding region, PCR reactions were performed using an extension time, allowing the amplification of genes without any insertion (between 600 and 700 bp), but not the mutated genes (more than 5 Kbp).

The analysis of the PCR products shows a single PCR product for the wild type and no PCR product for each transposon mutant, proving the presence of a transposon insert in the gene sequence for each of mutant analysed (Figure 5.1). Stocks of the confirmed transposon mutants were then generated and used to perform further experiments described in this chapter.

a



b

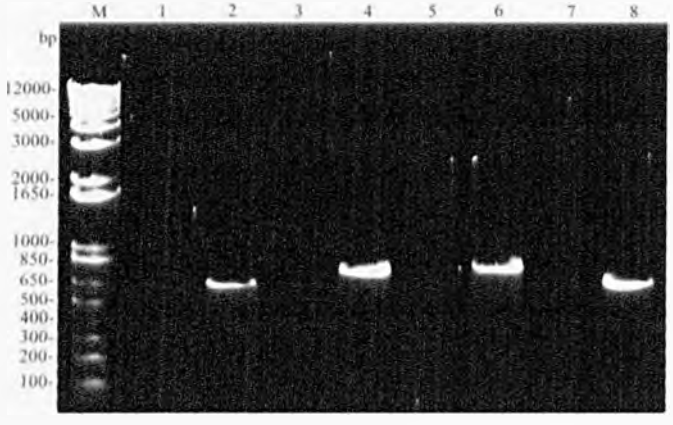


Figure 5.1 *P. aeruginosa* PAO1 single gene transposon mutants confirmation.
1% w/v agarose gel showing the azoreductase genes amplified from the total *P. aeruginosa* PAO1 wild type (wt) and single gene transposon mutants (trm) genomic DNA using gene specific primers. The gel shows the amplification of the azoreductase genes only for wild type strains, whereas no PCR products were obtained for any mutant (extension time in the PCR reaction allowed the amplification of the genes only their original and not in the mutated form). (**a**) 1 Kb Plus DNA Ladder (lane M), *paazor1* trm LacZ (lane 1), *paazor1* wt (lane 2), *paazor1* trm PhoA (lane 3), *paazor1* wt (lane 4), *paazor2* trm (lane 5), *paazor2* wt (lane 6), *paazor3* trm (lane 7), *paazor3* wt (lane 8), *pa0949* trm (lane 9), *pa0949* wt (lane 10). (**b**) *pa2580* trm (lane 1), *pa2580* wt (lane 2), *pa2280* trm (lane 3), *pa2280* wt (lane 4), *pa4975* trm (lane 5), *pa4975* wt (lane 6), *pa1204* trm (lane 7) and *pa1204* wt (lane 8).

5.2.2. Growth of *P. aeruginosa* PAO1 single gene transposon mutants

Analysis of planktonic growth was performed on *P. aeruginosa* PAO1 transposon mutants and compared with wild type. The results show that no growth differences were observed in the first six hours for any of the strains tested indicating that the exponential growth phase is very similar for all of them (Figure 5.2). From six hours of incubation the wild type starts the typical stationary phase and does not grow any further as determined by the absorbance at 600 nm for the entire time course. On the other hand, all the transposon mutants continued to grow for eight hours of incubation approximately. From this point, all the mutants show a stationary phase until the end of the time course. These data show a longer growth phase for each of the mutants compared with wild type strains and a higher growth rate between six and eight hours of incubation. However, statistical analyses show a significant difference only between wild type and the two mutants *paazor2* and *pa2280* ($p<0.05$) after eight hours and between *paazor2* and all the mutants except *pa2280* at 14 hours only. These data show only small differences for the mutant *paazor2* and only after the log phase, indicating that also for *paazor2* the growth pattern is very similar to wild type strain.

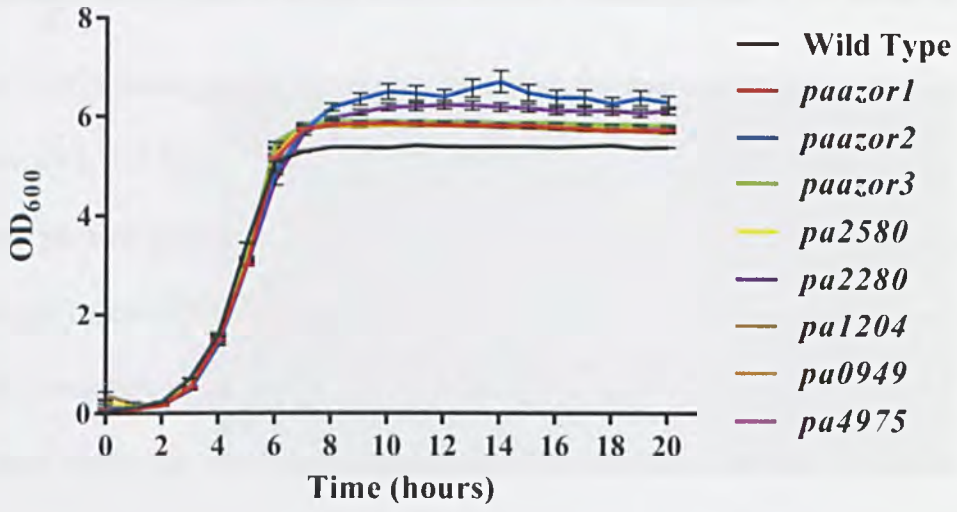


Figure 5.2 *P. aeruginosa* PAO1 wild type and azoreductase single gene transposon mutants growth analysis on LB broth.
Graph showing the wild type and azoreductase transposon mutants growth curves obtained in LB broth. All strains show same growth in the first six hours of incubation and similar growth between six and 20 hours. Bacterial strains were grown in 96 well plates at 37°C for 20 hours in the Inifite M200 PRO plate reader in triplicates and OD_{600} measurements were taken every 30 minutes. Statistical analysis (two-way ANOVA) shown significant difference between wild type and the two mutants *paazor2* after eight hours ($p<0.05$; significance $p<0.05$) and *pa2280* after ten hours ($p<0.05$; significance $p<0.05$); *paazor2* and all other mutants (except *pa2280*) at 14 hours only ($p<0.05$; significance $p<0.05$). Error bars showing the standard deviation of replicates (n=3).

5.2.3. Bacterial motility analysis of *P. aeruginosa* PAO1 single gene transposon mutants

Analysis of bacterial motility was performed by measuring the ability of wild type and transposon mutants to swarm on solid media such as LB, BHI and M9 salt agar (0.5% w/v).

On LB agar (Figure 5.3 **a**) all transposon mutants (colony size between 22.33 and 33 mm) shown significant higher motility ($p<0.0001$) than the wild type strain (16.50±3.47 mm). Amongst the mutants, *paazor1* (33±3.2 mm colonies size) shows the highest motility ($p<0.0001$), *pa1204* (22.33±2 mm colonies size) shows the lowest motility ($p<0.0001$), and all other show intermediate motility.

On BHI agar (Figure 5.3 **b**), the mutants shown less difference compared with the wild type strain, in fact only *paazor1* mutant (25.37±3.37 mm colonies size) shows a significant different motility ($p<0.05$) compared with wild type (19.63±1.51 mm colonies size), whereas all other mutants shown similar or lower motility that wild type strains, although no significance was found. Amongst mutants, only *paazor1* shows significant higher motility ($p<0.05$) than all other mutants (between 16.39 and 20.04 mm colonies size) except with *pa2580* (21.14±6.5 mm colonies size).

On M9 agar (Figure 5.3 **c**), again *P. aeruginosa* wild type (6.55±1.13 mm colony size) shown a lower motility than the transposon mutants. *pa2580* mutant shown the highest motility (11.05±2.62 mm colony size) and *pa0949* the lowest (7.93±0.82 mm colony size). However, no significant differences were found between wild type and any mutants or between the different mutants strains either, indicating that the swarming motility is the same for all strains tested on this medium.

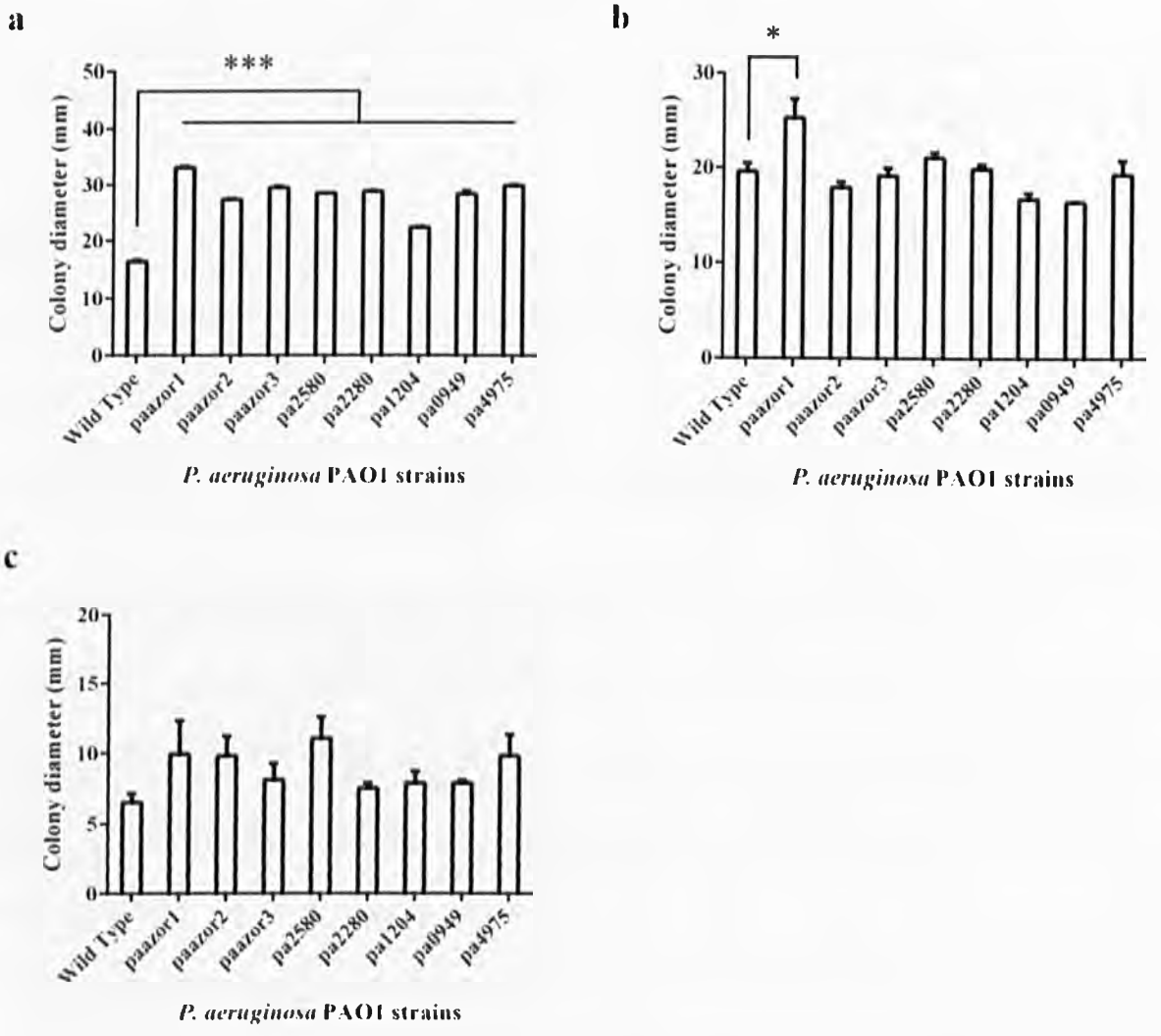


Figure 5.3 Motility analyses of *P. aeruginosa* PAO1 wild type and single gene transposon mutants on LB (a) BHI (b) and M9 salts (c) agar media.
Wild type and transposon mutant colony diameters obtained on different agar media (0.5% w/v). Bacterial overnight cultures were inoculated onto the medium surface and incubated at 37°C O/N before colony diameters were measured. Statistical analysis (one-way ANOVA) was performed on three replicates. Colony diameters obtained on (**a**) LB agar (**b**) BHI agar or (**c**) on M9 salts agar. Error bars showing the standard deviation of replicates (n=3).

5.2.4. Biofilm formation analysis of *P. aeruginosa* PAO1 single gene transposon mutants

In order to analyse the effect of mutating azoreductase genes on the *P. aeruginosa* PAO1 biofilm formation a series of experiments were performed to establish a robust method. These were carried out first using wild type strain only and then with the single gene transposon mutants also.

Two different methods, employing falcon tubes (Figure 5.4) and 96 well plates, were tested and the results are described here. The analysis of biofilm produced and attached to the cover slips using the falcon tube method shows the presence of biofilm on cover slips as a purple colour (Figure 5.5) with a high concentration on the cover slips at the border between the liquid an aerial phase in each tube. The quantification of biofilm was achieved by destaining showing that the biofilm produced by the wild type can be quantified approximately using this method (Figure 5.6). More biofilm was produced with longer incubation periods. However, this method did not appear robust or accurate enough as there was high variability amongst replicates.

P. aeruginosa PAO1 wild type biofilm production was then assessed in 96 well plates using similar methods, whereby the biofilm is quantified colorimetrically. The results obtained for the wild type strains show that high reproducibility was achieved with this method (Figure 5.7), and for this reason this was used to determine the biofilm production also for the single gene transposon mutants. It has also been observed that, using an initial inoculum such that the absorbance at 600 nm was between 0.3 and 0.4 gave the best results in terms of biofilm produced at incubation times of both 24 and 48 hours, and the biofilm produced after 48 hours of incubation was significantly higher than that produced after 24 hours ($p<0.01$). Subsequently, an initial absorbance value of 0.4 was chosen for all further experiments.

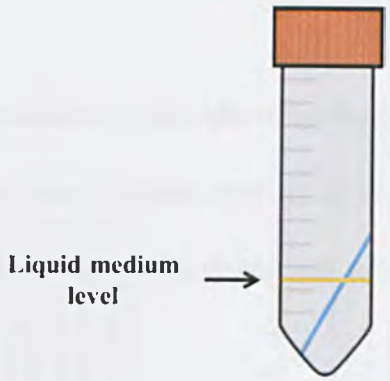


Figure5.4 Representation of *P. aeruginosa* PAO1 wild type biofilm production set up in falcon tubes.
Figure showing the representation of the biofilm production in falcon tubes. Microscope cover slips (blue) were placed into 50 ml falcon tubes containing 10 ml LB broth. The cover slips were only partially immerged into the liquid medium (yellow line representing the liquid level).

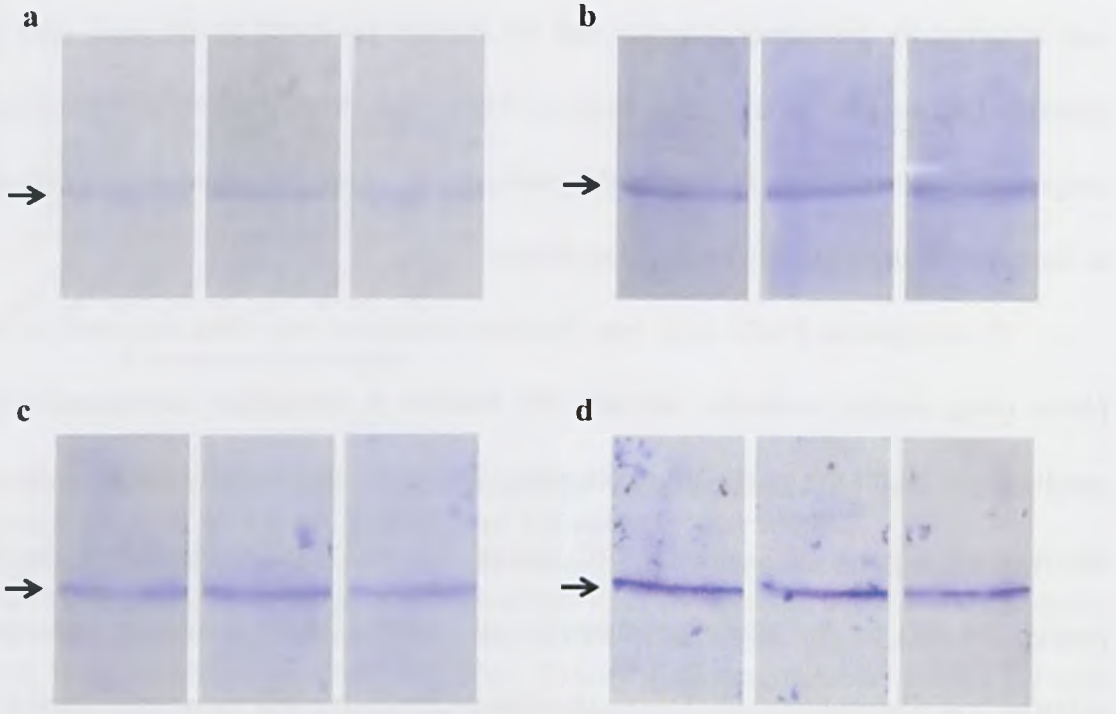


Figure 5.5 *P. aeruginosa* PAO1 wild type biofilm production on microscope cover slips.
Figure showing the *P. aeruginosa* PAO1 wild type biofilm produced (purple spots) after one (**a**), two (**b**), three (**c**) and four (**d**) hours. Wild type overnight cultures were used to inoculated 50 ml Falcon tubes containing 10 mL LB broth and sterile microscope cover slips. Samples were incubated at 37°C in static incubator between one and four hours. Cover slips were washed with ddH_2O and stained with 1% (v/v) crystal violet. Higher biofilm concentration was observed on the region between the liquid and aerial phases represented by a darker purple line (black arrows) and with the increasing of time as well.

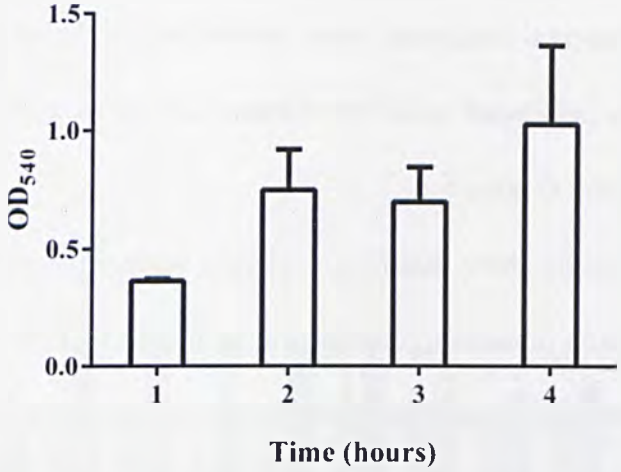


Figure 5.6 Quantification of *P. aeruginosa* PAO1 wild type biofilm produced on microscope cover slips in Falcon tubes.
Graph showing the quantification of wild type biofilm produced on coverslips after one, two, three and four hours. Wild type overnight cultures were used to inoculate 50 ml Falcon tubes containing 10 mL LB broth and sterile microscope cover slips. Samples were incubated at 37°C in static incubator between one and four hours. Cover slips were washed with ddH_2O, stained with 1% (v/v) crystal violet and distained with 95% (v/v) Ethanol. OD_{540} of distaining solutions were then measured. Higher biofilm concentration was observed with higher incubation times, although the statistical analysis (one-way ANOVA) shown no significant difference between samples. Error bars showing the standard deviation of three replicates (n=3).

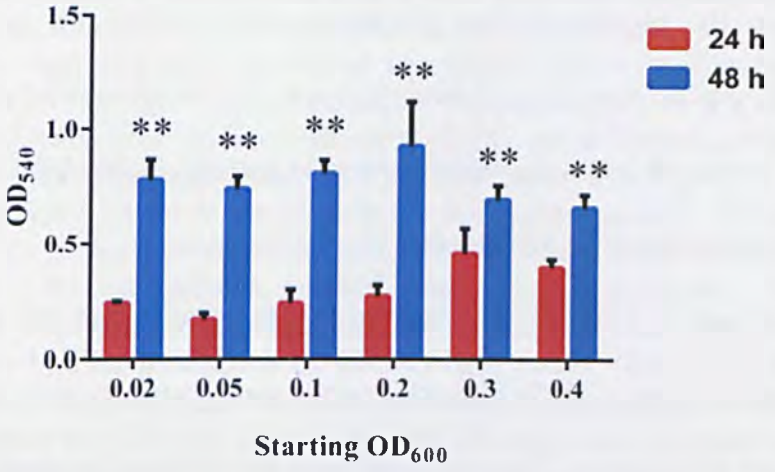


Figure 5.7 *P. aeruginosa* wild type biofilm production in 96 well plate.
Graph showing the wild type biofilm production after 24 and 48 hours incubation and using different starting OD_{600}. Wild type overnight cultures were used to inoculate 96 well plates containing 100 µl of LB broth in each well. Plates were incubated at 37°C in static incubator, washed with ddH_2O, stained with 1% (v/v) crystal violet and distained with 95% (v/v) Ethanol. OD_{540} of destaining solutions were then measured. Higher biofilm production was obtained using a starting OD_{600} of 0.3-0.4 with 24 hours and 0.2 with 48 hours incubation. Statistical analysis (t-test) shown significant higher biofilm production after 48 than after 24 hours ($p<0.01$). Error bars showing the standard deviation of three replicates (n=3).

Once the experimental conditions were established for the wild type strain, biofilm production analysis was performed on all transposon mutants as well as wild type using 24 and 48 hours of incubation (Figure 5.8).

The 24 hours results show that higher biofilm production (one or more fold) was obtained for all transposon mutants in comparison with wild type. In particular, significant difference ($p<0.05$) has been found between wild type and all mutants except *paazor2*, *paazor3* and *pa2580*. The mutants *pa2280* and *pa1204* were shown to be the highest and *paazor3* gave the lowest value, however with no significant differences between mutants.

After 48 hours incubation, higher biofilm production was also observed for the transposon mutants compared with wild type (between 20 and 50% more) but less than had been observed at 24 hours. Significantly higher biofilm production than wild type has been observed for the mutants *paazor1* and *paazor2* ($p<0.01$) showing approximately 50% higher biofilm production than wild type. Between transposon mutants, *paazor1* and *paazor2* shown the highest biofilm production and *pa0949* the lowest, however no significant differences have been found between any mutants. Comparison of biofilm production at 24 and 48 hours show that there is in general, higher biofilm production with a longer incubation period for all strains.

The difference between the two incubation periods was observed to be much greater for the wild type (200% higher). For the transposon mutants the difference was between 10 and 80% higher. This was confirmed by statistical analysis which shown no significant difference between 24 and 48 hours for some mutants (*pa2580*, *pa2280*, *pa1204*, *pa0949* and *pa4975*), whereas significance has been observed for *paazor1*, *paazor2* and *paazor3* as well as wild type. These data indicate that the transposon mutants and in particular *pa2580*, *pa2280*, *pa1204*, *pa0949* and *pa4975*, produce more biofilm than the wild type in the period up to 24 hours, whereas small or no differences are observed with longer incubation period between wild type and mutants.

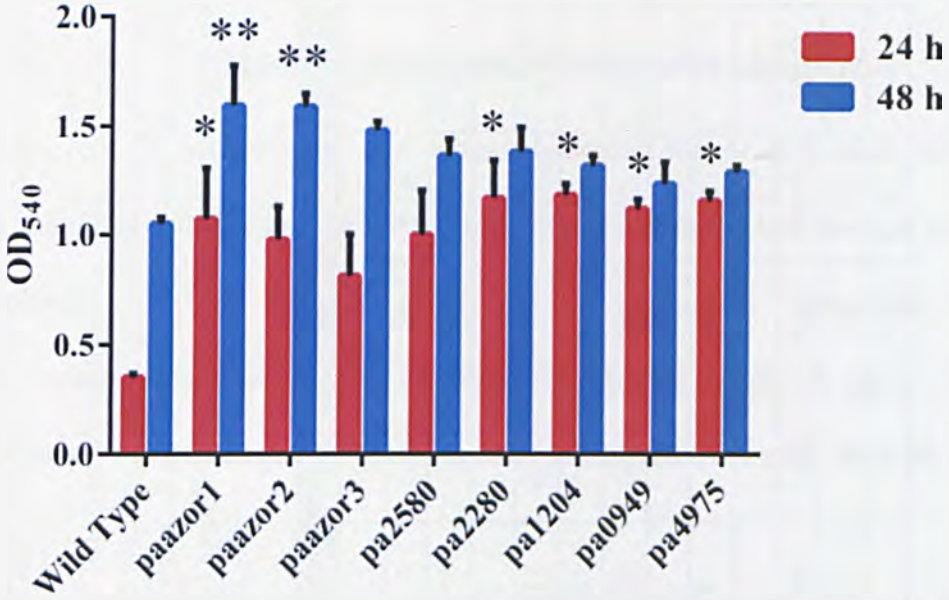


Figure 5.8 Comparison of *P. aeruginosa* PAO1 wild type and azoreductase single gene transposon mutants biofilm production after 24 and 48 hours.
Graph showing the wild type and azoreductase transposon mutants biofilm production after 24 (red bars) and 48 (blue bars) hours incubation. Statistical analysis (one-way ANOVA) of the biofilm production after 24 hours shows a significant difference between wild type and the transposon mutants *paazor1*, *pa2280*, *pa1204*, *pa0949* and *pa4975* ($p<0.05$), and no significant difference has been found between transposon mutants. Statistical analysis (one-way ANOVA) of the biofilm production after 48 hours shows a significant higher biofilm production for the mutants *paazor1* and *paazor2* compared with wild type ($p<.0.01$), and no significant difference has been found between transposon mutants. Comparison of the biofilm production at the two time points shows that higher biofilm production is always observed after 48 hours, however the difference between 24 and 48 hours is much higher in wild type (two fold) than all transposon mutants (0.1-0.8 fold), with *pa1204*, *pa0949* and *pa4975* mutants showing approximately the same amount of biofilm produced at the two time points (0.1 fold). Error bars showing the standard deviation of three replicates (n=3).

5.2.5. Antibiotic resistance analysis of *P. aeruginosa* PAO1 single gene transposon mutants

5.2.5.1. Minimal Inhibitory Concentration (MIC)

The sensitivity to antimicrobial agents was assessed for *P. aeruginosa* PAO1 transposon mutants and compared with wild type by determine MICs and using a range of different antibiotics belonging to the main families such as floroquinolones, aminoglycosides and beta lactams were used. Most antibiotic concentrations were used based on the MIC data available in the literature for *P. aeruginosa* (MacGowan and Wise, 2001).

The results obtained show that in all cases, except with meropenem, norfloxacin and novobiocin, different MIC values were obtained between wild and transposon mutants (Table 5.1). The transposon mutants show lower MICs than wild type with the fluoroquinolones (ciprofloxacin, levofloxacin and nalidixic acid) and colistin, whereas higher MICs were obtained with aminoglycosides (gentamicin and tobramycin). No MIC changes were observed with beta lactams.

Table 5.1 Minimal Inhibitory Concentration values (MIC) values of *P. aeruginosa* PAO1 wild type and azoreductase single gene transposon mutants using a range of antimicrobial agents.

Table showing the wild type and transposon mutants MIC values (μg/ml) obtained using fluoroquinolones, beta lactams, aminoglycosides and other antimicrobial agents. MICs obtained for transposon mutants were lower than wild type with fluoroquinolones, and in particular with the two antibiotics levofloxacin (1-127 fold) and ciprofloxacin (1-31 fold). Highly similar or same MICs between wild type and transposon mutants were obtained with beta lactams, aminoglycosides and other antimicrobial agents. MIC values were obtained using the microdilution method in 96 well plates.

Antibiotic Class	Antibiotic	wild type	*paazor1*	*paazor2*	*paazor3*	*pa2580*	*pa2280*	*pa1204*	*pa0949*	*pa4975*
Fluoroquinolones	Ciprofloxacin	1	0.13	0.06	0.25	0.06	0.03	0.06	0.5	0.06
	Levofloxacin	4	0.13	0.06	0.25	0.06	0.03	0.06	0.5	0.06
	Nalidixic acid	500	250	250	250	250	250	250	250	500
	Norfloxacin	4	4	4	4	4	4	4	4	4
Beta lactams	Meropenem	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
	Piperacillin	8	8	8	8	8	8	8	8	8
Aminoglycosides	Gentamicin	0.47	0.94	0.94	1.88	0.94	0.94	7.5	0.94	1.88
	Tobramycin	1	1	1	1	2	2	2	1	2
Others	Ceftazidime	2	4	4	4	4	4	8	4	4
	Colistin	8	2	2	2	2	2	2	2	2
	Novobiocin	1000	1000	1000	1000	1000	1000	1000	1000	1000
	Rifampicin	25	25	25	25	25	25	25	25	50
	Tigecycline	4	4	4	4	2	4	4	4	4

The MIC results obtained with fluoroquinolones show that lower MIC values were found for all mutants than for wild type when levofloxacin, ciprofloxacin and nalidixic acid are used, whereas same MICs between wild type and mutants were found with norfloxacin. The treatment with levofloxacin resulted to be the more effective in inhibiting the growth of the mutants. In fact, the mutant MICs were 0.03 µg/ml (*pa2280*), 0.06 µg/ml (*paazor2*, *pa2580*, *pa1204* and *pa4975*), 0.13 µg/ml (*paazor1*), 0.25 µg/ml (*paazor3*) and 0.5 µg/ml (*pa0949*) in comparison with the 4 µg/ml obtained for wild type. A considerable different MIC values between wild type (1 µg/ml) and transposon mutants (between 0.03 and 0.5 µg/ml) were also obtained with ciprofloxacin and between wild type (500 µg/ml) and transposon mutants (between 250 and 500 µg/ml) with nalidixic acid. This data indicate that the antibiotic concentration needed to inhibit the bacterial growth is lower for transposon mutants than wild type when this class of antibiotic is used, and in particular levofloxacin and ciprofloxacin.

The results obtained with beta lactams shown same MICs for wild type and transposon mutants with meropenem and piperacillin indicating that there are no differences in sensitivity when the azoreductase genes are knocked out.

With aminoglycoside antibiotics, a different trend was observed. Higher MIC values were found for all transposon mutants than for the wild type strain: in particular, with gentamicin, the mutants for the genes *pa1204*, *paazor2* and *pa4975* shown a considerable higher MIC (7.5, 1.88 and 1.88 µg/ml respectively) than wild type (0.47 µg/mL). This indicates that mutation of these genes increase the resistance to aminoglycosides and to gentamicin in particular in relation to growth inhibition.

The treatments with other antimicrobial agents not belonging to the classes previously described shown a mix of results. Treatments with colistin shown lower MIC (2 µg/ml) for all mutants than wild type (8 µg/ml). From the other end, treatments with ceftazidime shown higher MIC for all mutants (between 4 and 8 µg/ml) than wild type (2

μg/ml). The other three antibiotic tested (norfloxacin, rifampicin and tigecycline) didn't show any particular difference between wild type and transposon mutants.

For a direct MIC comparison between wild type and transposon mutants and to establish the increase/decrease of sensitivity to antibiotic treatments for the transposon mutants, the ratio of wild type and mutant MICs (MIC_{WT}/MIC_{mutant}) were calculated. In this way the fold change between MIC_{mutant} and MIC_{WT} was obtained showing values above one for $MIC_{WT}>MIC_{mutant}$ indicating an increase of sensitivity for transposon mutant strains and below one for $MIC_{WT}<MIC_{mutant}$ indicating an increase of resistance for transposon mutant strains. This analysis was performed only with the most effective antimicrobial agents showing a lower MIC for transposon mutants (levofloxacin, ciprofloxacin, colistin, nalidixic acid and tigecycline).

The MICs comparisons shows an increase of sensitivity for all transposon mutants (Figure 5.9). The highest increase of sensitivity was observed with the mutant *pa2280* (127 fold) followed by *paazor2*, *pa2580*, *pa1204* and *pa4975* (63 fold) when treated with levofloxacin. A lower effect, but still considerable, was also obtained for the mutants *paazor1* (31 fold), *paazor3* (15 fold) and *pa0949* (7 fold). Also with ciprofloxacin, colistin and nalidixic acid treatments an increase of sensitivity was observed for all mutants (1-33, 4 and 1 fold respectively), however levofloxacin shown to be the most effective fluoroquinolone.

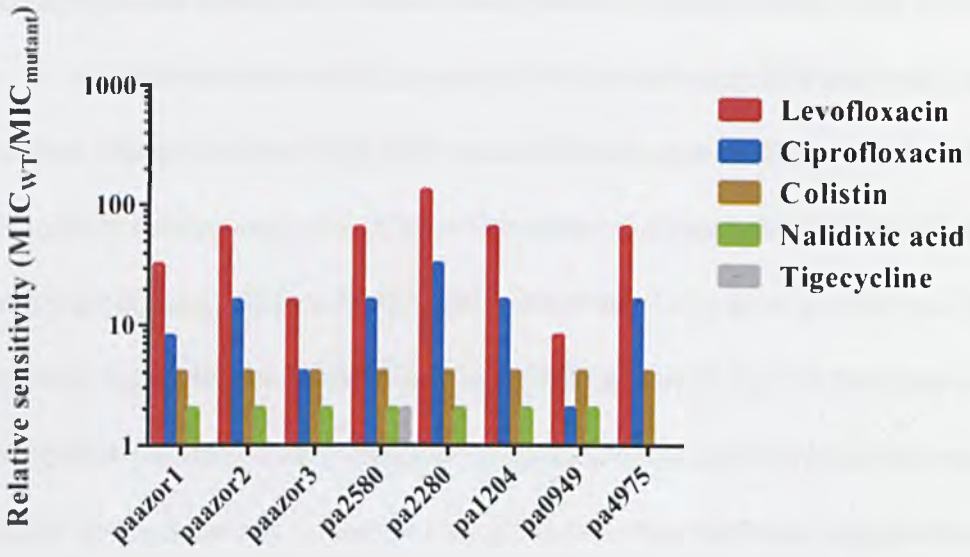


Figure 5.9 Representation of *P. aeruginosa* PAO1 azoreductase single gene transposon mutants increase of sensitivity.
Graph showing the azoreductase transposon mutants increase of sensitivity relative to wild type strain. The data shown here are based on the comparison of MICs for wild type and mutants (MIC_{WT}/MIC_{mutant}), which represent the change in antibiotic sensitivity of all transposon mutants. The graph includes only the antibiotics for which an increase of sensitivity was observed in the transposon mutants, showing that all transposon mutants are more sensitive than wild type to fluoroquinolones treatments, in particular with levofloxacin (31-127 fold) and ciprofloxacin (1-31 fold).

5.2.5.2. Minimal Bactericidal concentration (MBC)

In order to determine the bactericidal effect of some antibiotics used to determine the MIC, MBC assays were performed on *P. aeruginosa* PAO1 wild type and transposon mutants. To do this, bacterial cultures obtained from MIC determination were used to inoculate LB agar plates as shown in Figure 5.10. For this analysis the most effective antibiotics such as levofloxacin and ciprofloxacin were chosen, as well as some other representing all the antibiotic families tested with the MIC analysis.

The MBC analysis (Table 5.2) shows a range of different results from the MIC data, showing that all transposon mutant strains exhibit a lower MBC than the wild type with levofloxacin. The most affected strains were *paazor2* and *paazor3* transposon mutants, which shown an MBC 7 fold lower than wild type. Mutants for the genes *paazor1*, *pa2580* and *pa0949* show a MBC 3 fold lower than wild type, whereas *pa2280* and *1204* mutants are only 1 fold more sensitive than wild type. These data indicate that all the mutant strains, and in particular *paazor2* and *paazor3* are killed at a lower antibiotic concentration than wild type. Regarding the other fluoroquinolones tested, ciprofloxacin, the transposon mutants *paazor1* (3 fold), *paazor2* (3 fold), *pa1204* (1 fold), *pa0949* (1 fold) and *pa4975* (1 fold) show a lower MBC than wild type, whereas *paazor3*, *pa2580* and *pa2280* have a higher MBC (1 fold) than wild type.

The MBC values obtained with tobramycin show that for all transposon mutants the MBC is 8 fold lower than wild type, except for *pa1204* mutants (3 fold). Also in this case, the transposon mutants exhibit an increased sensitivity compared with wild type.

The results obtained with meropenem illustrate that all transposon mutants exhibit a higher MBC than wild type (between 1 and 7 fold) indicating a lower sensitivity to this antibiotic. No MBC variation between wild type and transposon mutants was observed when colistin and tigecycline were used.

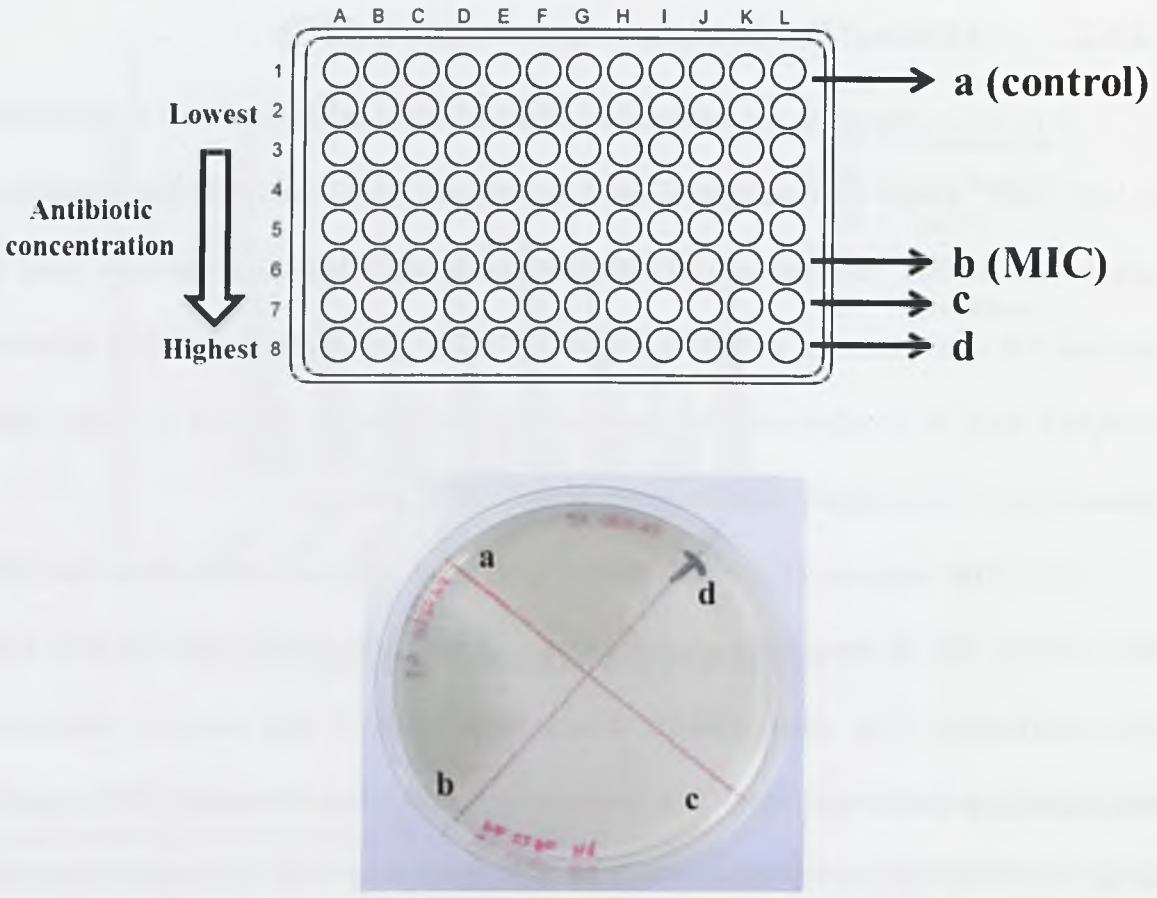


Figure 5.10 Example of Minimal Bactericidal Concentration (MBC) determination on *P. aeruginosa* PAO1 single gene transposon mutant *pa2280*.
Picture showing the MBC determination of *pa2280* transposon mutants carried out with bacterial samples from MIC test performed using tobramycin. Following the MIC determination, positive control (**a**), MIC (**b**) and higher concentrations (**c** and **d**) were used to inoculate LB agar plates containing 12.5 µg/mL tetracycline. The lowest concentration used in the MIC test, and in which bacterial growth has not been shown on plates, was considered as MBC for that specific antibiotic. Plates were inoculated with 50 µL taken from the appropriate wells of the MIC determination 96 well plate and streaked on LB agar plates divided in four sections.

Table 5.2 Minimal Bactericidal Concentration (MBC) determined for *P. aeruginosa* PAO1 wild type and single gene azoreductase transposon mutants.

The table shows the MBC values (μg/ml) obtained for wild type and transposon mutants using a selection on antibiotic including fluoroquinolones, beta lactams, aminoglycosides and others. The data obtained show a higher sensitivity for the mutants compare with wild type with levofloxacin (1-7 fold), ciprofloxacin (1-3 fold) and (3-7 fold) tobramycin, whereas same or lower sensitivity than wild type was obtained with meropenem, colistin and tigecycline. Data were generated as illustrated and described in figure 5.10.

Antibiotic Class	Antibiotic	Wild Type	*paazor1*	*paazor2*	*paazor3*	*pa2580*	*pa2280*	*pa1204*	*pa0949*	*pa4975*
Fluoroquinolones	Ciprofloxacin	2	0.5	0.5	4	4	4	1	1	1
	Levofloxacin	8	2	1	1	2	4	4	2	4
Beta lactams	Meropenem	0.5	4	2	4	4	4	4	1	1
Aminoglycosides	Tobramycin	8	1	1	1	1	1	2	1	1
Others	Colistin	32	32	32	32	32	32	32	32	32
	Tigecycline	80	80	80	80	80	80	80	80	80

5.3. Discussion

The aims described in section 1.7 have been met as follows:

Analysis of *P. aeruginosa* PAO1 swarming motility and biofilm production using azoreductase single gene transposon mutants

a. Analysis of azoreductase genes role on swarming motility, biofilm production and infection in *P. aeruginosa* PAO1

Analysis of P. aeruginosa PAO1 antibiotic resistance using azoreductase single gene transposon mutants

a. Analysis of azoreductase genes role on inhibition (MIC) and viability (MBC) of *P. aeruginosa* PAO1 by antimicrobial agents

Since the introduction of antibiotics in clinical therapy, bacteria have developed increasingly more sophisticated resistance strategies. This has led to the appearance and spread of the so-called 'superbugs', resistant to practically all antimicrobial drugs available on the market.

The Gram negative bacterium *P. aeruginosa*, which can infect a wide range of animal and plant hosts has become a superbug (Rahme et al., 1995). It is a leading cause of nosocomial infections, as well as chronic lung infections in CF patients, causes serious infections of burn wounds and eye lesions and causes systemic infections of immunocompromised individuals with a high mortality rate (Mutlu and Wunderink, 2006, Holder, 1993, Lyczak et al., 2000, Govan and Deretic, 1996). For these reason it has become extremely important to find new antimicrobial target for antimicrobial agents in *P. aeruginosa*.

In order to study the physiological role of *P. aeruginosa* PAO1 azoreductase and

putative azoreductase genes, and to verify the hypothesis for which they may be used as new antimicrobial target, single gene transposon mutants were used. These were checked using a PCR-based approach and it has been shown the presence of the mutations in the correct genes into the gene coding region. In this way the *P. aeruginosa* PAO1 strains with disrupted azoreductase and putative azoreductase genes could be used to evaluate the effect of the absence of these genes, and consequently the corresponding proteins, on the bacterial physiology. This was analysed performing a series of experiments including growth, motility and biofilm analysis as well as evaluation of antibioatic resistance.

The bacterial growth analysis shown that there are no significant differences between wild type and transposon mutants, although recent studies shown that *paazor1* may be involved in the bacterial growth processes (Rakhimova et al., 2008). The same gene has been shown to be also involved in the infection process of this pathogen (Wiehlmann and Tummler, 2007), however experiments conducted on lettuce (data not shown) have not shown any difference between wild type and *paazor1* mutant as well as all other mutants, suggesting that no effects on pathogenesis can be correlated to these genes.

P. aeruginosa it has also become increasingly problematic due to its ability to adapt to and thrive in a variety of environmental niches by means of virulence factors such as formation of biofilms that provide protection from both toxic and antibacterial compounds in the environment, as well as predation (Matz and Kjelleberg, 2005, Fujitani et al., 2011). In this study, the ability to produce biofilm by azoreductase and putative azoreductse mutants was tested over a period of time of 48 hours. The data obtained indicate that in a short period (24 hours) all mutants show higher biofilm production compare with wild type. This difference then disappears with a longer incubation period (48 hours) suggesting that the biofilm production process is concentrated in a shorter incubation period for azoreductase and putative azoreductase mutants than wild type. This is particularly evident

for the mutants *pa2580*, *pa2280*, *pa1204*, *pa0949* and *pa4975*, which do not show an increase of biofilm production after 24 hours. In terms of amount of biofilm produced, all transposon mutants shown higher production than wild type, however no significant differences have been obtained between them, indicating that azoreductase and putative azoreductase genes may not be involved in biofilm production in this bacterium.

The swarming ability is another important environmental adaptation associated with virulent *P. aeruginosa* strains. Swarming colonies exhibit a greater antibiotic resistance as well as expression of virulence factor compare to planktonic growth (Overhage et al., 2008, Lai et al., 2009). The swarming analysis shows that all transposon mutants exhibit higher motility than the wild type in relatively rich medium such as LB. These data indicate that these genes may be involved in the swarming activity of *P. aeruginosa*. However, the results obtained on M9-salt and BHI, indicate that in extreme conditions (very poor or rich environments) these genes do not have any effect on the motility process. For this reason, the hypothesis for which azoreductase genes may be involved in this process is not very strong.

It has been shown that motility plays an important role in colonization of different environments, attachment of the bacteria to surfaces and biofilm formation in *P. aeruginosa* (O'Toole and Kolter, 1998). Considering this, strains showing higher motility may exhibit also higher biofilm formation. Comparing the motility and the 24 hours biofilm production results, both obtained on the same medium LB, similar difference between wild type and transposon mutants can be observed. In fact, the transposon mutants shown higher swarming activity as well as higher biofilm production than wild type strains. Although the high similarity between motility and biofilm production patterns, there is no direct correlation between level of swarming and biofilm production. For example, one of the most motile mutants (*pa1204*) shows one of the highest biofilm production. Based on this and considering that the 48 hours biofilm production results

shown that the differences between wild type and mutants occurs only in the first 24 hours, it can be hypothesized that also in the case of swarming the difference between wild type and transposon mutants may disappear in a longer time period, suggesting that azoreductase and putative azoreductase genes may be not involved in these two physiological processes.

Compared with other pathogens, *P. aeruginosa* is very difficult to eradicate as it displays high intrinsic resistance to a wide variety of antibiotics (Poole, 2002). In addition, these intrinsic mechanisms can be stabilized or enhanced by mutation, which can lead to acquisition of new resistance, and resulting in MDR strains. All of these resistance mechanisms are part of the genetic makeup of this pathogen, leading to very high baseline MICs and making many common antibiotics ineffective.

Considering the multiple ways in which *P. aeruginosa* is resistant, it is not surprising that resistance can be observed for all currently available anti-pseudomonal antibiotics. Although antibiotics are available and usable for most *P. aeruginosa* infections, resistance rates are on the rise and its high intrinsic resistance makes it very challenging to find new drugs. Previous data shown that *P. aeruginosa* PAO1 *pa2580* mutant exhibits between 2 and 3 fold higher sensitivity to chloramphenicol, ciprofloxacin and carbenicillin (Chen et al., 2010b). Because the gene *pa2580* has been shown to have the same biochemical function of all other genes considered in this study, it was thought that also the other azoreductase and putative azoreductase genes may have an effect on antibiotic sensitivity. To prove this, the effect of mutating azoreductase and putative azoreductase genes on antibiotic resistance was analysed by comparing MICs and MBCs between wild type and transposon mutants.

The MIC data obtained show that all transposon mutants used exhibit an increase in sensitivity to fluoroquinolones treatments, whereas much less or no increases have been observed with all other antibiotics tested, including beta lactams and aminoglycosides.

Additionally, the increase of sensitivity observed here was extremely higher than what has been previously shown. Although all mutants show a high increase of sensitivity to fluoroquinolone treatments, *pa2280* exhibits the greatest effect (127 fold with levofloxacin and 31 fold with ciprofloxacin) in comparison with wild type.

The increase of sensitivity to fluoroquinolones of all mutants was also confirmed by MBCs analysis, showing an increase up to 7 and 3 fold with levofloxacin and ciprofloxacin respectively. Interestingly, also with tobramycin, which have not shown a particular difference from during the MIC analysis, the transposon mutants shown an increase of sensitivity up to 8-fold compare with wild type. This indicate that these genes are important for the survival of *P. aeruginosa* under treatment with the most common antibiotics (fluoroquinolones and aminoglycosides), as well as growth of this bacterium under fluoroquinolone treatments. These results clearly show the effect of mutating azoreductase genes on the bacterial antibiotic resistance process, emphasizing the importance of these genes in the contest of *P. aeruginosa* infection treatments.

Chapter 6. Conclusions and future work

The aims of the work presented in this thesis have been towards understanding of azoreductases and putative azoreductases identified in *P. aeruginosa* PAO1. Understanding the role of these genes and their products was in turn directed towards gaining insight into whether these gene products would be a good target for chemical inhibition. This, as stated previously, constitutes one of the major health concerns for people suffering from CF. In this chapter I expand on discussion of the original aims of the study as stated in chapter 1.

6.1. Sequencing and cloning of *P. aeruginosa* PAO1 putative azoreductase genes

In chapter 3, the sequencing and cloning of five putative azoreductase genes has been described. Whilst 10 azoreducatse genes have been identified of which three (*paazor1*, *paazor2* and *paazor3*) have already been cloned (Wang et al., 2007, Ryan et al., 2010b), in chapter 3 I describe the cloning of a further five genes (*pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*). In addition, the remaining genes (*pa1224* and *pa1225*) have subsequently been cloned by Sinaed Holland. Thus the genes *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975* have been sequenced and shown to correspond exactly to the published gene sequence in the Pseudomonas Genome Database (Winsor et al., 2011).

These gene were cloned into pET28b(+) within its multiple restriction cloning sites, which has the inducible promoter *lacUV5* sequence at its 5' end and a polyhistisine tag coding sequence at its 3' end. The constructs generated were then transfected into *E. coli* JM109ES and BL21 strains. Positive clones were screened via PCR of individual colonies and confirmed by sequencing, showing also in this case the identity between cloned and published gene sequences.

6.2. Production and purification of *P. aeruginosa* PAO1 putative azoreductases as recombinant proteins

Chapter 3 also describes the production and purification of PA0949, PA1204, PA2280 and PA2580 recombinant proteins. Over-expression of the genes *pa0949*, *pa1204*, *pa2280*, *pa2580* was achieved by inducing the *lacUV5* promoter with IPTG. Following induction with IPTG, proteins were extracted and purified using Ni-NTA matrix (IMAC).

The yield of pure soluble proteins, varied from 30 to 50 mg/l of culture, was produced using 500 ml of culture for PA0949, PA1204 and PA2580 and a series of smaller scale cultures for PA2280. Interestingly, all proteins were likely to be soluble based on their sequences rather than membrane bound proteins and each of the recombinant proteins could be concentrated to at least 2 mg/ml in the aqueous buffer.

Mass spectrometry analysis of each of the purified protein show high correspondence between obtained and predicted protein molecular weight (±1%).

The presence of a hexahistidine tag on each protein sequence was shown by western blot and thrombin digestion (decrease of protein molecular weights after thrombin digestion) and loss of reactivity with the hexahistisine antibody.

The remaining putative azoreductase proteins (PA1224, PA1225 and PA4975) have been subsequently purified by Sinead Holland and the yields found for these proteins (between 10 and 30 mg/l of culture) show that they are also highly soluble.

6.3. Biochemical and enzymatic analyses of *P. aeruginosa* PAO1 putative azoreductase recombinant proteins

In chapter 4 biochemical and enzymatic assays in relation to the presence of flavin molecules, establishment of the condition of enzymatic reactions and substrate specificity for the purified PA0949, PA1204, PA2280 and PA2580 recombinant proteins are described.

Analysis of the presence of flavin molecules shows that these can be detected via protein spectra and TLC only for the recombinant protein PA1204 and PA2580 (FAD in both cases). Regarding the conditions of enzymatic reduction of substrates by these proteins, it has been shown that PA1204 and PA2580 display higher reduction rates in presence of FAD, whereas for PA0949 and PA2280 FMN is the preferred flavin.

In terms of reductant, NADPH was shown to be the preferred reductant for each of the enzymes. Additionally, for the protein PA0949, enzymatic activity has been observed only in presence of FMN and NADPH, making this combination essential for detecting the activity of this protein. These data also show that all purified recombinant proteins are active, allowing further enzymatic analyses.

Substrate specificity of all recombinant proteins was assessed using a range of azo compounds and quinones. The data obtained show that the proteins PA1204, PA2280 and PA2580 are able to reduce both azo compounds and quinones and therefore they have been proved to the same family of the previous characterized enzymes (Ryan et al., 2014), whereas PA0949 has been shown to reduce only quinones. As previously shown for paAzoR1, paAzoR2 and pAazoR3 proteins (Wang et al., 2007, Ryan et al., 2010b, Ryan et al., 2014) quinone oxidoreductase activities is in all cases is higher than the azoreductase activity.

Structural studies would be useful to clarify if flavin molecules are bound and the mode of binding to these proteins and clarify the mode of binding of reductants to azoreductases and the basics of reductant selectivity by these proteins.

The work presented in chapters 3 and 4 paves the way for investigating inhibitors as the tools for looking for inhibitors are now available. The possibility that known classes of drugs could act as inhibitors of azoreductases, including antibiotics, is something which should be explored now that the pure proteins can be produced.

6.4. Analysis of *P. aeruginosa* PAO1 biofilm production and swarming motility using azoreductase single gene transposon mutants

Chapter 5 describs the effect of mutating azoreductase genes on biofilm production and swarming motility in *P. aeruginosa* PAO1. These assays were performed using single gene transposon mutants for the azoreductase genes *paazor1*, *paazor2*, *paazor3*, *pa0949*, *pa1204*, *pa2280*, *pa2580* and for the putative azoreductase gene *pa4975* as well as the wild type strain (without mutations). Each mutant consists of a *P. aeruginosa* PAO1 strain with an insertion of a transposon element (*lacZ* or *phoA*) into the gene coding sequence (Jacobs et al., 2003). In this way, each mutant results in the disruption of the synthesis of one azoreductase or putative azoreductase protein.

The eight mutants used in this study were checked via PCR prior use and for all of them, the insertion was confirmed to be in the correct gene. Analysis of planktonic growth of mutants and wild type showed no difference between any of the strains used.

Swarming motility was analysed on different media and it showed that all mutants display higher motility than wild type only on a relatively rich medium (LB), whereas no differences were observed on very rich or poor media (BHI and M9-salt respectively).

Similar results have been obtained for biofilm production in LB broth medium, where the mutants show higher biofilm production than wild type. However, this was observed after 24 hours incubation period, whereas no significant differences have been observed after 48 hours of incubation, suggesting that mutation of azoreductase and putative azoreductase genes may affect the rate of biofilm production across a shorter time period.

Although the biomass of biofilm produced was measured, no information on biofilm structure and viability were collected. To do this, approaches such as confocal microscopy and LIVE/DEAD cell viability assays could be applied. Further investigation on biofilm production such as in presence of antibiotics would be an interesting possibility

to investigate to improve the understanding of the role of azoreductase genes in this process.

6.5. Analysis of *P. aeruginosa* PAO1 antibiotic resistance using azoreductase single gene transposon mutants

Chapter 5 also describes the effect of mutating azoreductase genes on antibiotic resistance in *P. aeruginosa* PAO1. Also in this case mutants for azoreductase and putative azoreductase genes (*paazor1*, *paazor2*, *paazor3*, *pa0949*, *pa1204*, *pa2280*, *pa2580* and *pa4975*) were used. To test the effect of antibiotics on mutants and wild type, MICs and MBCs were determined.

MICs determination was performed using a range of different antibiotics belonging to the main antibiotic families. Results show that, with a good number of antibiotics, all mutants exhibit a higher growth inhibition than wild type. This was observed in particular with fluoroquinolones, which show a mutant growth inhibition up to 127 fold higher than wild type. Amongst the fluoroquinolones, levofloxacin was demonstrated to be the most effective antibiotic and the mutant for the gene *pa2280* the most sensitive (at least 2 times more sensitive than the other mutants).

MBC analysis was performed using a selection of the antibiotics used for the MIC determination representing each of antibiotic family. It was also found in the MBC study that the mutants were more sensitive than wild type to antibiotic treatments. Levofloxacin shown to be one of the most effective antibiotic and the mutants for the genes *paazor2* and *paazor3* the most sensitive (7 fold more sensitive than wild type).

Although MIC results obtained with aminoglycosides did not show any relevant difference between mutants and wild type, the MBC data related to tobramycin show the same effect obtained with levofloxacin on all azoreductase and putative azoreductase mutants (7 fold more sensitive than wild type).

These results strongly suggest that azoreductase and putative azoreductase genes or

their products may be involved in the growth inhibition process as well as bacterial survival during antibiotic treatments.

Although the presence of the mutations has been verified using a PCR-based approach, this method has not given a precise information on the location of the insertion. An alternative method, such as the complete sequencing of the mutated genes, could be applied in order to confirm the mutations as well as establish the exact position of the insertion in each gene.

The effect of mutating azoreductase genes on expression and/or regulation of other genes is another area that could be explored in order to improve the understanding of the function of azoreductase genes in *P. aeruginosa*. This could be carried out using gene expression approaches such as qRT-PCR for a limited number of genes or NGS (New Generation Sequencing) for a genome-scale approach.

Complementation of azoreductase mutants is ongoing at the moment. This will allow repetition of the antibiotic resistance studies (MICs and MBCs determination) and verify whether the wild type phenotype could be restored for each mutant following complementation to add further information to understanding whether the sensitivity to antibiotics has a correlation with azoreductases.

Over expression of azoreductase enzymes using non tetracycline resistant plasmid in *E. coli* strains would be another interesting experiment to perform. This could be done using tetracycline like antibiotics in order to test the antibiotic resistance role of azoreductase genes with this class of antimicrobial agents.

Whether the azoreductase proteins can directly inactivate antibiotics is another question that needs to be answered. This could be done by performing new enzymatic assays using antibiotics, and fluoroquinolones in particular, as substrate.

APPENDIX I

Alignment of *pa2580* sequence from *P. aeruginosa* PAO1 (FWD and REV) and the Pseudomonsa database (pa2580)

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             TGGTGATGGC TCGCTGCGGG TAGAGTTCGT GCCGGTCATC GAGGGCTGGC
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    55         65         75         85         95
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             TGCGTGGGGG CGCAATGAAG ACGCGCATCC CAGCTCTTCT GGCGATTCGT
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   105        115        125        135        145
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             GAGTGCGGAT CAAATCTGTT TTACCGCCGT ACGACTTTTT CCGTTCCCTG
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   155        165        175        185        195
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             CGCCTATCTA ACCTGCCGCT CTCTTCAACC CGGGATCGGC AGGCATATCC
REV             ---------- ---------- ---------- --GGATCGGC AGGCATATCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   205        215        225        235        245
pa2580          ---------- ---------- --------AT GAAAAACATT CTCCTGCTCA
FWD             GCTGCGGTTC CAGCATGTAC GGAACACCAT GAAAAACATT CTCCTGCTCA
REV             GCTGCGGTTC CAGCATGTAC GGAACACCAT GAAAAACATT CTCCTGCTCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   255        265        275        285        295
pa2580          ACGGCGGAAA GCGTTTCGCC CATTCCGACG GTCGCCTCAA CCAGACCCTG
FWD             ACGGCGGAAA GCGTTTCGCC CATTCCGACG GTCGCCTCAA CCAGACCCTG
REV             ACGGCGGAAA GCGTTTCGCC CATTCCGACG GTCGCCTCAA CCAGACCCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345
pa2580          CACGAAACCG CCCTGGCCCA TCTGGACCGC CGTGGCTTCG ACCTGCGCCA
FWD             CACGAAACCG CCCTGGCCCA TCTGGACCGC CGTGGCTTCG ACCTGCGCCA
REV             CACGAAACCG CCCTGGCCCA TCTGGACCGC CGTGGCTTCG ACCTGCGCCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa2580          GACCTTCATC GACGGTGGCT ATGATATCCC GACGGAGGTG GACAAGTTCC
FWD             GACCTTCATC GACGGTGGCT ATGATATCCC GACGGAGGTG GACAAGTTCC
REV             GACCTTCATC GACGGTGGCT ATGATATCCC GACGGAGGTG GACAAGTTCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa2580          TCTGGGCCGA CGTGGTGATC TACCAGATGC CCGGCTGGTG GATGGGCGCC
FWD             TCTGGGCCGA CGTGGTGATC TACCAGATGC CCGGCTGGTG GATGGGCGCC
REV             TCTGGGCCGA CGTGGTGATC TACCAGATGC CCGGCTGGTG GATGGGCGCC
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
pa2580          CCGTGGACAG TGAAGCGCTA CATCGACGAA GTCTTCACGG CCGGACACGG
FWD             CCGTGGACAG TGAAGCGCTA CATCGACGAA GTCTTCACGG CCGGACACGG
REV             CCGTGGACAG TGAAGCGCTA CATCGACGAA GTCTTCACGG CCGGACACGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
pa2580          CAGCCTCTAT GCCAACGACG GTCGTACCCG CTCCGACAGC ACGCAGAAAT
FWD             CAGCCTCTAT GCCAACGACG GTCGTACCCG CTCCGACAGC ACGCAGAAAT
REV             CAGCCTCTAT GCCAACGACG GTCGTACCCG CTCCGACAGC ACGCAGAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
pa2580          ATGGCAGCGG CGGTCTGGTG CAGGGCAAGC GCTACATGAT CTCGGCGACC
FWD             ATGGCAGCGG CGGTCTGGTG CAGGGCAAGC GCTACATGAT CTCGGCGACC
REV             ATGGCAGCGG CGGTCTGGTG CAGGGCAAGC GCTACATGAT CTCGGCGACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
pa2580          TGGAATGCAC CACGGCAGGC GTTCGACGAT CCGAGCGACT TCTTCGAAGG
FWD             TGGAATGCAC CACGGCAGGC GTTCGACGAT CCGAGCGACT TCTTCGAAGG
REV             TGGAATGCAC CACGGCAGGC GTTCGACGAT CCGAGCGACT TCTTCGAAGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   655        665        675        685        695
pa2580          AAAAGGCGTG GATGCGGTGT ATTTCCCCTT CCACAAGGCC AACCAGTTCC
FWD             AAAAGGCGTG GATGCGGTGT ATTTCCCCTT CCACAAGGCC AACCAGTTCC
REV             AAAAGGCGTG GATGCGGTGT ATTTCCCCTT CCACAAGGCC AACCAGTTCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   705        715        725        735        745
pa2580          TCGGCATGTC CGGCCTGCCG ACGTTCCTCG CGGTGGATGT GATGAAGCGC
FWD             TCGGCATGTC CGGCCTGCCG ACGTTCCTCG CGGTGGATGT GATGAAGCGC
REV             TCGGCATGTC CGGCCTGCCG ACGTTCCTCG CGGTGGATGT GATGAAGCGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   755        765        775        785        795
pa2580          CCGGACGTCC CGGCTACCGT CGCTGCCTAC CAGGCGCACC TGGACAGGGT
FWD             CCGGACGTCC CGGCTACCGT CGCTGCCTAC CAG-CG---- ----------
REV             CCGGACGTCC CGGCTACCGT CGCTGCCTAC CAGGCGCACC TGGACAGGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   805        815        825        835        845
pa2580          GTTCGGGCGC GCCGGCTGA- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             GTTCGGGCGC GCCGGCTGAG TTCGACGGCG GGCCTCGTGT CGCTTCGCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   855        865        875        885        895
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             GATGGAGTGG CGGGTCGAAA TCCAGGCTCT CGGGACGAGG CCTAATGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   905        915        925        935        945
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             ACGGATCCGC TATCCGGGCT TTTCTCGGCC ACGACCCGCG AGTGATCCGA
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                   955        965        975        985        995
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             AATTCGATTT TTCCATCGAT CCGTCGAATT AGATTTCATC GGGGACGGGC

                ....|....| ....|....| ....|....| ....|..
                  1005       1015       1025       1035
pa2580          ---------- ---------- ---------- -------
FWD             ---------- ---------- ---------- -------
REV             AGGAAAGGAT GCTTGCACGA ATATCGAATG GCGCATC
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Alignment of *pa2280* sequence from *P. aeruginosa* PAO1 (FWD and REV) and the Pseudomonsa database (pa2280)

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                   5          15         25         35         45
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             GGGGAGCCTG CCCGCTGTAT CTCGGCGCAG CGCTGAAGGC CCATTGGGGC
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   55         65         75         85         95
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             CTGGCCGATC CCTCCGCCCT GGATGGCGAC GAAGCCCTGC GGGATGCGGC
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                  105        115        125        135        145
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             GTTCCACGCC ACCCTGGCAC GCATCGAACA GCGTTGCCGA GCCTTCCTCG
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                  155        165        175        185        195
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             GCCTGCCCTT CGCTACCCTG GATCGCGACC AGCTCAAGCG TGAGCTGGAG
REV             ---------- ------CCTG GATCGCGACC AGCTCAAGCG TGAGCTGGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                  205        215        225        235        245
pa2280          ---------- ---------- ---------A TGTCCGAACA ACTACCCAAC
FWD             CGCATCGGCT CGCTCTGACC GGAGGAAGCA TGTCCGAACA ACTACCCAAC
REV             CGCATCGGCT CGCTCTGACC GGAGGAAGCA TGTCCGAACA ACTACCCAAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                  255        265        275        285        295
pa2280          CTCGATCCCG CGCTGCTCGG CGACCCGCCC CCCGTCTCCG GGCACAGGCC
FWD             CTCGATCCCG CGCTGCTCGG CGACCCGCCC CCCGTCTCCG GGCACAGGCC
REV             CTCGATCCCG CGCTGCTCGG CGACCCGCCC CCCGTCTCCG GGCACAGGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                  305        315        325        335        345
pa2280          GCGCATCCTC CTGCTCTACG GCTCGACCCG CGAGCGCTCC TTCAGCCGCC
FWD             GCGCATCCTC CTGCTCTACG GCTCGACCCG CGAGCGCTCC TTCAGCCGCC
REV             GCGCATCCTC -TGCTCTACG GCTCGACCCG CGAGCGCTCC TTCAGCCGC-
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                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa2280          TGCTGGTGCT GGAGGCCGCA CGCCTGCTCG AACGCTTCGG TGCCGAAACG
FWD             TGCTGGTGCT GGAGGCCGCA CGCCTGCTCG AACGCTTCGG TGCCGAAACG
REV             TGCTGGTGCT GGAGGCCGCA CGCCTGCTCG AACGCTTCGG TGCCGAAACG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa2280          CGGATTTTCG ACCCTTCCGG GCTGCCATTG CCCGATGATG CACCGGTGGA
FWD             CGGATTTTCG ACCCTTCCGG GCTGCCATTG CCCGATGATG CACCGGTGGA
REV             CGGATTTTCG ACCCTTCCGG GCTGCCATTG CCCGATGATG CACCGGTGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
pa2280          GCATCCCAAG GTCCGCGAGT TGCGCGACCT GGTGCAGTGG TCGGAAGGCC
FWD             GCATCCCAAG GTCCGCGAGT TGCGCGACCT GGTGCAGTGG TCGGAAGGCC
REV             GCATCCCAAG GTCCGCGAGT TGCGCGACCT GGTGCAGTGG TCGGAAGGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
pa2280          AGGTCTGGTG CTCGCCCGAG CGCCACGGTG CGCTGTCCGC GGTATTCAAG
FWD             AGGTCTGGTG CTCGCCCGAG CGCCACGGTG CGCTGTCCGC GGTATTCAAG
REV             AGGTCTGGTG CTCGCCCGAG CGCCACGGTG CGCTGTCCGC GGTATTCAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
pa2280          GCGCAGATCG ACTGGATTCC CCTGGCACTC GGGGCGGTGC GCCCGACCCA
FWD             GCGCAGATCG ACTGGATTCC CCTGGCACTC GGGGCGGTGC GCCCGACCCA
REV             GCGCAGATCG ACTGGATTCC CCTGGCACTC GGGGCGGTGC GCCCGACCCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
pa2280          GGGCAAGACC CTGGCGCTGA TGCAGGTCTG CGGCGGCTCG CAGTCGTTCA
FWD             GGGCAAGACC CTGGCGCTGA TGCAGGTCTG CGGCGGCTCG CAGTCGTTCA
REV             GGGCAAGACC CTGGCGCTGA TGCAGGTCTG CGGCGGCTCG CAGTCGTTCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   655        665        675        685        695
pa2280          ACGTGGTCAA CCAGTTGCGC GTGCTGGGTC GCTGGATGCG CATGTTCACC
FWD             ACGTGGTCAA CCAGTTGCGC GTGCTGGGTC GCTGGATGCG CATGTTCACC
REV             ACGTGGTCAA CCAGTTGCGC GTGCTGGGTC GCTGGATGCG CATGTTCACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   705        715        725        735        745
pa2280          ATCCCCAACC AGTCCTCGGT TCCCAAGGCC TACCTGGAGT TCGACGAAGC
FWD             ATCCCCAACC AGTCCTCGGT TCCCAAGGCC TACCTGGAGT TCGACGAAGC
REV             ATCCCCAACC AGTCCTCGGT TCCCAAGGCC TACCTGGAGT TCGACGAAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   755        765        775        785        795
pa2280          GGGCCGGATG AAGCCATCGC CCTACTACGA CCGGGTGGTC GACGTGATGG
FWD             GGGCCGGATG AAGCCATCGC CCTACTACGA CCGGGTGGTC GACGTGATGG
REV             GGGCCGGATG AAGCCATCGC CCTACTACGA CCGGGTGGTC GACGTGATGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   805        815        825        835        845
pa2280          AGGAGTTGTT CAAGTTCACC CTGCTCCTGC GCGAGCGCAC GGACTTCCTG
FWD             AGGAGTTGTT CAAGTTCACC CTGCTCCTGC GCGAGCGCAC GGACTTCCTG
REV             AGGAGTTGTT CAAGTTCACC CTGCTCCTGC GCGAGCGCAC GGACTTCCTG
```

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                   855        865        875        885        895
pa2280          GTGGACCGCT ATTCGGAGCG CAAGGAAAGC GCCGAGCAGC TTTCCGCGCG
FWD             GTGGAC---- ---------- ---------- ---------- ----------
REV             GTGGACCGCT ATTCGGAGCG CAAGGAAAGC GCCGAGCAGC TTTCCGCGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   905        915        925        935        945
pa2280          GGTCGACCAG CGTTCGCTCT GA-------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             GGTCGACCAG CGTTCGCTCT GAACCGCGTC CGCCTCACTC GCGCCCCGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   955        965        975        985        995
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             GGATGGCCGA AGCGCGCGCG GTAGGCATGG GTGAAGTTTT CCGGACTGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                  1005       1015       1025       1035       1045
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             GTAACCCAGG CGATAGGCTA TCTGCGCGAT CTGCCAGCGA CCCTCGCGGA

                ....|....| ....|....| ....
                  1055       1065
pa2280          ---------- ---------- ----
FWD             ---------- ---------- ----
REV             CCATCTGCCG GCCGATCTCG AGAC
```

Alignment of *pa1204* sequence from *P. aeruginosa* PAO1 (FWD and REV) and the Pseudomonsa database (pa1204)

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45
pa1204          ---------- ---------- ---------- ---------- ----------
FWD             AGTGCCGCTG GAGGGTATCG ACGTGCAACT GAACATCGTC GAGGAACGCA
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    55         65         75         85         95
pa1204          ---------- ---------- ---------- ---------- ----------
FWD             CCAAGCCGGA GCCCTTCACC CATATCCGCC GCGACATCCG CCTGGCCGGC
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   105        115        125        135        145
pa1204          ---------- ---------- ---------- ---------- ----------
FWD             GATCTCGACG CCGAGCAGCG CCAGCGTCTG CTGGAGATCG CCAATGCCTG
REV             ---------- ---------- -------CTG CTGGAGATCG CCAATGCCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   155        165        175        185        195
pa1204          ---------- ---------- ---------- ---------- ----------
FWD             CCCGATCCAT CGTGTACTCT CCGGAGAGGT CAGCGTCAGC AGCCAGCTAC
REV             CCCGATCCAT CGTGTACTCT CCGGAGAGGT CAGCGTCAGC AGCCAGCTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                205        215        225        235        245
pa1204     ---------- ---------- ---------- -----ATGAG CGACGACATC
FWD        GCGACTGAAC CCGCACGCAC AACGAGGAGC ACGGCATGAG CGACGACATC
REV        GCGACTGAAC CCGCACGCAC AACGAGGAGC ACGGCATGAG CGACGACATC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                255        265        275        285        295
pa1204     AAGGTATTGG GCATTTCCGG CAGCCTGCGC AGCGGCTCCT ACAACAGCGC
FWD        AAGGTATTGG GCATTTCCGG CAGCCTGCGC AGCGGCTCCT ACAACAGCGC
REV        AAGGTATTGG GCATTTCCGG CAGCCTGCGC AGCGGCTCCT ACAACAGCGC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                305        315        325        335        345
pa1204     GGCGCTGCAG GAGGCGATTG GCCTGGTCCC GCCGGGCATG AGCATCGAGC
FWD        GGCGCTGCAG GAGGCGATTG GCCTGGTCCC GCCGGGCATG AGCATCGAGC
REV        GGCGCTGCAG GAGGCGATTG GCCTGGTCCC GCCGGGCATG AGCATCGAGC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                355        365        375        385        395
pa1204     TGGCGGACAT CTCCGGCATC CCGCTGTACA ACGAGGACGT CTACGCCCTC
FWD        TGGCGGACAT CTCCGGCATC CCGCTGTACA ACGAGGACGT CTACGCCCTC
REV        TGGCGGACAT CTCCGGCATC CCGCTGTACA ACGAGGACGT CTACGCCCTC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                405        415        425        435        445
pa1204     GGCTTCCCGC CCGCGGTGGA ACGCTTCCGC GAGCAGATTC GCGCGGCGGA
FWD        GGCTTCCCGC CCGCGGTGGA ACGCTTCCGC GAGCAGATTC GCGCGGCGGA
REV        GGCTTCCCGC CCGCGGTGGA ACGCTTCCGC GAGCAGATTC GCGCGGCGGA

           ....|....| ....|....| ....|....| ....|....| ....|....|
                455        465        475        485        495
pa1204     CGCGCTGCTG TTCGCCACCC CGGAATACAA CTATTCGATG GCCGGGGTGC
FWD        CGCGCTGCTG TTCGCCACCC CGGAATACAA CTATTCGATG GCCGGGGTGC
REV        CGCGCTGCTG TTCGCCACCC CGGAATACAA CTATTCGATG GCCGGGGTGC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                505        515        525        535        545
pa1204     TGAAGAATGC CATCGACTGG GCCTCGCGGC CGCCGGAGCA GCCGTTCTCC
FWD        TGAAGAATGC CATCGACTGG GCCTCGCGGC CGCCGGAGCA GCCGTTCTCC
REV        TGAAGAATGC CATCGACTGG GCCTCGCGGC CGCCGGAGCA GCCGTTCTCC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                555        565        575        585        595
pa1204     GGCAAGCCGG CGGCGATCCT CGGCGCCAGC GCCGGGCGTT TCGGCACCGC
FWD        GGCAAGCCGG CGGCGATCCT CGGCGCCAGC GCCGGGCGTT TCGGCACCGC
REV        GGCAAGCCGG CGGCGATCCT CGGCGCCAGC GCCGGGCGTT TCGGCACCGC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                605        615        625        635        645
pa1204     GCGGGCGCAG TATCACTTGC GCCAGACGCT GGTGTTCCTC GACGTTCATC
FWD        GCGGGCGCAG TATCACTTGC GCCAGACGCT GGTGTTCCTC GACGTTCATC
REV        GCGGGCGCAG TATCACTTGC GCCAGACGCT GGTGTTCCTC GACGTTCATC

           ....|....| ....|....| ....|....| ....|....| ....|....|
                655        665        675        685        695
pa1204     CGCTGAACAA GCCGGAAGTG ATGATCTCCA GCGCGCAGAA CGCCTTCGAT
FWD        CGCTGAACAA GCCGGAAGTG ATGATCTCCA GCGCGCAGAA CGCCTTCGAT
REV        CGCTGAACAA GCCGGAAGTG ATGATCTCCA GCGCGCAGAA CGCCTTCGAT

           ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                 705         715         725         735         745
pa1204      GCCCAGGGCC GGCTGCTCGA CGACAAGGCG CGCGAGCTGA TCCAGCAGCA
FWD         GCCCAGGGCC GGCTGCTCGA CGACAAGGCG CGCGAGCTGA TCCAGCAGCA
REV         GCCCAGGGCC GGCTGCTCGA CGACAAGGCG CGCGAGCTGA TCCAGCAGCA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 755         765         775         785         795
pa1204      GTTGCAGGCC CTGCAGCTAT GGGTGCGCCG CCTGCGCGGT TGA-------
FWD         GTTGCAGGCC CTGCAGCTAT GGGTGCGCCG CCTGCGCGGT TGAATGCCTC
REV         GTTGCAGGCC CTGCAGCTAT GGGTGCGCCG CCTGCGCGGT TGAATGCCTC

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 805         815         825         835         845
pa1204      ---------- ---------- ---------- ---------- ----------
FWD         GTCACCGGAG AGCCTGAACA TGAGCAATAC CGAAGAGCGT TGCGACCTGT
REV         GTCACCGGAG AGCCTGAACA TGAGCAATAC CGAAGAGCGT TGCGACCTGT

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 855         865         875         885         895
pa1204      ---------- ---------- ---------- ---------- ----------
FWD         CCTCC----- ---------- ---------- ---------- ----------
REV         CCTCCTCGCG GGACTGTCCG GAACGCCACG AACACCTGCT GCAACGGGTC

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 905         915         925         935         945
pa1204      ---------- ---------- ---------- ---------- ----------
FWD         ---------- ---------- ---------- ---------- ----------
REV         ACCGCGCGTG CCGCGGAAAT CGGCGGCGGG ATCACCGTCA GTCGGCTGAT

            ....|....| ....|....| ....|....| ....|....| ....|
                 955         965         975         985         995
pa1204      ---------- ---------- ---------- ---------- -----
FWD         ---------- ---------- ---------- ---------- -----
REV         GCCGTCGCGC CAGCGGCGGA TGATCGGTGC CTGGTGCTCC TCGAA
```

Alignment of *pa0949* sequence from *P. aeruginosa* PAO1 (FWD and REV) and the Pseudomonsa database (pa0949)

```
            ....|....| ....|....| ....|....| ....|....| ....|....|
                 5           15          25          35          45
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         GGACGGGGAG GACGAATACA AGGCCCTCGG CCTGGATGAG CCCAGTCTTG
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 55          65          75          85          95
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         GCGAAGAGGC CATCATCGAG GCAATGGCCA GGCATCCGAA GCTGATCGAG
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 105         115         125         135         145
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         CGCCCGGTGC TGGTCGCGGG TGACAAGGCG GTGGTTGGCC GGCCTCCGGA
REV         ---------- ---------- --ACAAGGCG GTGGTTGGCC GGCCTCCGGA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                 155         165         175         185         195
```

```
pa0949          ---------- ---------- -TTGAGCAGT CCCTACATCC TGGTGCTGTA
FWD             AAGAGTCCTG GAGATCCTCG CTTGAGCAGT CCCTACATCC TGGTGCTGTA
REV             AAGAGTCCTG GAGATCCTCG CTTGAGCAGT CCCTACATCC TGGTGCTGTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   205        215        225        235        245
pa0949          CTACAGTCGC CATGGCGCTA CCGCGGAAAT GGCCCGGCAG ATCGCCCGTG
FWD             CTACAGTCGC CATGGCGCTA CCGCGGAAAT GGCCCGGCAG ATCGCCCGTG
REV             CTACAGTCGC CATGGCGCTA CCGCGGAAAT GGCCCGGCAG ATCGCCCGTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   255        265        275        285        295
pa0949          GCGTCGAACA GGGCGGCTTC GAGGCGCGTG TACGCACGGT TCCCGCGGTA
FWD             GCGTCGAACA GGGCGGCTTC GAGGCGCGTG TACGCACGGT TCCCGCGGTA
REV             GCGTCGAACA GGGCGGCTTC GAGGCGCGTG TACGCACGGT TCCCGCGGTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345
pa0949          TCCACCGAAT GCGAAGCGGT CGCCCCCGAC ATCCCCGCGG AGGGGGCGCT
FWD             TCCACCGAAT GCGAAGCGGT CGCCCCCGAC ATCCCCGCGG AGGGGGCGCT
REV             TCCACCGAAT GCGAAGCGGT CGCCCCCGAC ATCCCCGCGG AGGGGGCGCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa0949          GTACGCAACC CTGGAGGACC TGAAGAACTG CGCGGGCCTG GCCCTCGGCA
FWD             GTACGCAACC CTGGAGGACC TGAAGAACTG CGCGGGCCTG GCCCTCGGCA
REV             GTACGCAACC CTGGAGGACC TGAAGAACTG CGCGGGCCTG GCCCTCGGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa0949          GCCCGACCCG CTTCGGCAAC ATGGCTTCCC CGCTGAAATA CTTCCTCGAC
FWD             GCCCGACCCG CTTCGGCAAC ATGGCTTCCC CGCTGAAATA CTTCCTCGAC
REV             GCCCGACCCG CTTCGGCAAC ATGGCTTCCC CGCTGAAATA CTTCCTCGAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
pa0949          GGTACCAGCA GCCTGTGGCT GACCGGCAGC CTGGTCGGCA AGCCGGCAGC
pa0949 FWD      GGTACCAGCA GCCTGTGGCT GACCGGCAGC CTGGTCGGCA AGCCGGCAGC
REV             GGTACCAGCA GCCTGTGGCT GACCGGCAGC CTGGTCGGCA AGCCGGCAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
pa0949          GGTCTTCACC TCCACCGCCA GCCTGCACGG CGGCCAGGAG ACCACTCAGT
FWD             GGTCTTCACC TCCACCGCCA GCCTGCACGG CGGCCAGGAG ACCACTCAGT
REV             GGTCTTCACC TCCACCGCCA GCCTGCACGG CGGCCAGGAG ACCACTCAGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
pa0949          TATCGATGCT GTTGCCATTG CTGCACCACG GCATGCTGGT CCTGGGCATT
FWD             TATCGATGCT GTTGCCATTG CTGCACCACG GCATGCTGGT CCTGGGCATT
REV             TATCGATGCT GTTGCCATTG CTGCACCACG GCATGCTGGT CCTGGGCATT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
pa0949          CCCTACAGCG AACCCGCCCT GCTGGAAACC CGCGGCGGCG GCACGCCTTA
FWD             CCCTACAGCG AACCCGCCCT GCTGGAAACC CGCGGCGGCG GCACGCCTTA
REV             CCCTACAGCG AACCCGCCCT GCTGGAAACC CGCGGCGGCG GCACGCCTTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   655        665        675        685        695
```

```
pa0949      CGGCGCCAGC CACTTCGCCG GCGCCGATGG CAAGCGCAGC CTCGATGAGC
FWD         CGGCGCCAGC CACTTCGCCG GCGCCGATGG CAAGCGCAGC CTCGATGAGC
REV         CGGCGCCAGC CACTTCGCCG GCGCCGATGG CAAGCGCAGC CTCGATGAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   705        715        725        735        745
pa0949      ACGAACTGAC CCTGTGTCGC GCGCTGGGCA AACGCCTGGC GGAAACCGCC
FWD         ACGAACTGAC CCTGTGTCGC GCGCTGGGCA AACGCCTGGC GGAAACCGCC
REV         ACGAACTGAC CCTGTGTCGC GCGCTGGGCA AACGCCTGGC GGAAACCGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   755        765        775        785        795
pa0949      GGCAAGCTGG GGAGTTGA-- ---------- ---------- ----------
FWD         GGCAAGCTGG GGAGTTGAAA TGGCCCGCAA GAACAAACCG CTGCCCGAAG
REV         GGCAAGCTGG GGAGTTGAAA TGGCCCGCAA GAACAAACCG CTGCCCGAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   805        815        825        835        845
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         TAGCGTGGCT GCAGCCGCGC CTGGCGCTCA GCCGCGCCAT CAGCCTGGGA
REV         TAGCGTGGCT GCAGCCGCGC CTGGCGCTCA GCCGCGCCAT CAGCCTGGGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   855        865        875        885        895
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         AGCTTCGTCG GCCTGGTCCT GCTC------ ---------- ----------
REV         AGCTTCGTCG GCCTGGTCCT GCTCATCCTC ATTCGCGACC TGATCTACGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   905        915        925        935        945
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         ---------- ---------- ---------- ---------- ----------
REV         GGACGCCCAT GGTGCGGCGA GCTGGGTGCC CTGGCTGGTC CTCGCCTTCA

                ....|....| ....|....| ....|....| ....|....| ..
                   955        965        975        985
pa0949      ---------- ---------- ---------- ---------- --
FWD         ---------- ---------- ---------- ---------- --
REV         AGCTGCTGCC GCTGCTGGTG GTGGCCCCCG GACTGCTCAT GG
```

Alignment of *pa0949* sequence from *P. aeruginosa* PAO1 (FWD and REV) and the Pseudomonsa database (pa4975)

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45
pa4975      ---------- ---------- ---------- ---------- ----------
FWD         TGCGCCGGAC GGCGGCATGT TCGTGATGGT CGACATCCGT CCCACCGGAC
REV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    55         65         75         85         95
pa4975      ---------- ---------- ---------- ---------- ----------
FWD         TTTCCGCCCA GGCCTTCGCC GACCGCCTGC TGGACCGCCA TGGCGTATCG
REV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   105        115        125        135        145
pa4975      ---------- ---------- ---------- ---------- ----------
FWD         GTGCTCGCCG GCGAAGCCTT CGGCCCGAGC GCCGCCGGTC ACATCCGCCT
```

```
REV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   155        165        175        185        195
pa4975      ---------- ---------- ---------- ---------- ----------
FWD         CGGCCTGGTG TTGGGCGCCG AACCGCTGCG CGAAGCCTGT CGGCGCATTG
REV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   205        215        225        235        245
pa4975      ---------- ---------- ---------- ---------- ----------
FWD         CGCTGTGCGC CGCCGAGTTG CTCGGCCAGG CCTGAACCCC TCGGCCGCGA
REV         ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   255        265        275        285        295
pa4975      ---------- ---------- ---------- -----ATGAA CGTACTGATC
FWD         GCGATGCGGC CGGTCACCAC GATTCGGAAA CCACGATGAA CGTACTGATC
REV         ---------- CGGTCACCAC GATTCGGAAA CCACGATGAA CGTACTGATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345
pa4975      GTCCACGCCC ACAACGAACC GCAATCCTTC ACCCGCGCGC TCTGTGACCA
FWD         GTCCACGCCC ACAACGAACC GCAATCCTTC ACCCGCGCGC TCTGTGACCA
REV         GTCCACGCCC ACAACGAACC GCAATCCTTC ACCCGCGCGC TCTGTGACCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa4975      GGCATGCGAG ACCCTGGCAG GCCAGGGCCA CGCGGTGCAG GTCTCGGATC
FWD         GGCATGCGAG ACCCTGGCAG GCCAGGGCCA CGCGGTGCAG GTCTCGGATC
REV         GGCATGCGAG AGCCTGGCAG GCCAGGGCCA CGCGGTGCAG GTCTCGGATC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa4975      TCTACGCGAT GAACTGGAAT CCGGTGGCCA GTGCCGCCGA CTTCGCCGAG
FWD         TCTACGCGAT GAACTGGAAT CCGGTGGCCA GTGCCGCCGA CTTCGCCGAG
REV         TCTACGCGAT GAACTGGAAT CCGGTGGCCA GTGCCGCCGA CTTCGCCGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
pa4975      CGCGCCGATC CCGACTACCT GGTGTACGCC CTGGAGCAGC GCGAGAGCGT
FWD         CGCGCCGATC CCGACTACCT GGTGTACGCC CTGGAGCAGC GCGAGAGCGT
REV         CGCGCCGATC CCGACTACCT GGTGTACGCC CTGGAGCAGC GCGAGAGCGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
pa4975      CAAGCGCCAG AGCCTGGCCG CCGACATCCA GGCCGAGCTG GACAAGCTGC
FWD         CAAGCGCCAG AGCCTGGCCG CCGACATCCA GGCCGAGCTG GACAAGCTGC
REV         CAAGCGCCAG AGCCTGGCCG CCGACATCCA GGCCGAGCTG GACAAGCTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
pa4975      TGTGGGCCGA CCTGCTGATC CTCAACTTTC CGATCTACTG GTTCTCGGTG
FWD         TGTGGGCCGA CCTGCTGATC CTCAACTTTC CGATCTACTG GTTCTCGGTG
REV         TGTGGGCCGA CCTGCTGATC CTCAACTTTC CGATCTACTG GTTCTCGGTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
pa4975      CCGGCGATCC TCAAGGGGTG GTTCGACCGG GTACTGGTGT CCGGGGTCTG
FWD         CCGGCGATCC TCAAGGGGTG GTTCGACCGG GTACTGGTGT CCGGGGTCTG
```

```
REV          CCGGCGATCC TCAAGGGGTG GTTCGACCGG GTACTGGTGT CCGGGGTCTG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                655        665        675        685        695
pa4975       CTATGGCGGC AAGCGCTTCT ACGACCAGGG TGGCCTGGCC GGCAAGAAGG
FWD          CTATGGCGGC AAGCGCTTCT ACGACCAGGG TGGCCTGGCC GGCAAGAAGG
REV          CTATGGCGGC AAGCGCTTCT ACGACCAGGG TGGCCTGGCC GGCAAGAAGG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                705        715        725        735        745
pa4975       CGCTGGTCAG CCTGACCCTG GGCGGGCGCC AGCACATGTT CGGCGAGGGT
FWD          CGCTGGTCAG CCTGACCCTG GGCGGGCGCC AGCACATGTT CGGCGAGGGT
REV          CGCTGGTCAG CCTGACCCTG GGCGGGCGCC AGCACATGTT CGGCGAGGGT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                755        765        775        785        795
pa4975       GCCATCCACG GACCGCTGGA GGACATGCTG CGGCCGATCC TGCGCGGCAC
FWD          GCCATCCACG GACCGCTGGA GGACATGCTG CGGCCGATCC TGCGCGGCAC
REV          GCCATCCACG GACCGCTGGA GGACATGCTG CGGCCGATCC TGCGCGGCAC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                805        815        825        835        845
pa4975       CCTGGCCTAT GTCGGCATGC AGGTGCTGGA GCCCTTCGTC GCCTGGCACG
FWD          CCTGGCCTAT GTCGGCATGC AGGTGCTGGA GCCCTTCGTC GCC-------
REV          CCTGGCCTAT GTCGGCATGC AGGTGCTGGA GCCCTTCGTC GCCTGGCACG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                855        865        875        885        895
pa4975       TGCCATACAT CAGCGAGGAA GCGCGCGGCA ACTTCCTGCG CGCCTACCGG
FWD          ---------- ---------- ---------- ---------- ----------
REV          TGCCATACAT CAGCGAGGAA GCGCGCGGCA ACTTCCTGCG CGCCTACCGG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                905        915        925        935        945
pa4975       GCGCGGCTGG AAAATCTCGA GCAGGATGTA CCCCTGCGGT TCCCGCGGCT
FWD          ---------- ---------- ---------- ---------- ----------
REV          GCGCGGCTGG AAAATCTCGA GCAGGATGTA CCCCTGCGGT TCCCGCGGCT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                955        965        975        985        995
pa4975       GGAGCAGTTC GACGCGCTGC TCCAGCCGCT GGCGCGCTGA ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          GGAGCAGTTC GACGCGCTGC TCCAGCCGCT GGCGCGCTGA CCCGGGCGAC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                1005       1015       1025       1035       1045
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          GCGCGCCAGG CCAGTCAAGC CAAGACGCCC CCGACCGGGG GCGTCTTGGC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                1055       1065       1075       1085       1095
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          TCGTGCTAGT ACTGCTGGCG CGGCTTGCTC TGTAACTGCT CGGCGGCGGC

             ....|....| ....|....| ....|....| ....|....| ....|....|
                1105       1115       1125       1135       1145
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
```

```
REV          CTTGGCCAGG TCCGGCGGGA GGAAGTCCTT GTCCGGATCG TAGTCCTGCT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                1155       1165       1175       1185       1195
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          TCAGGTAGGC GCTGAGCGCC TCCAGGTCGG CCGGGCTGAG GGTGCCGCCG

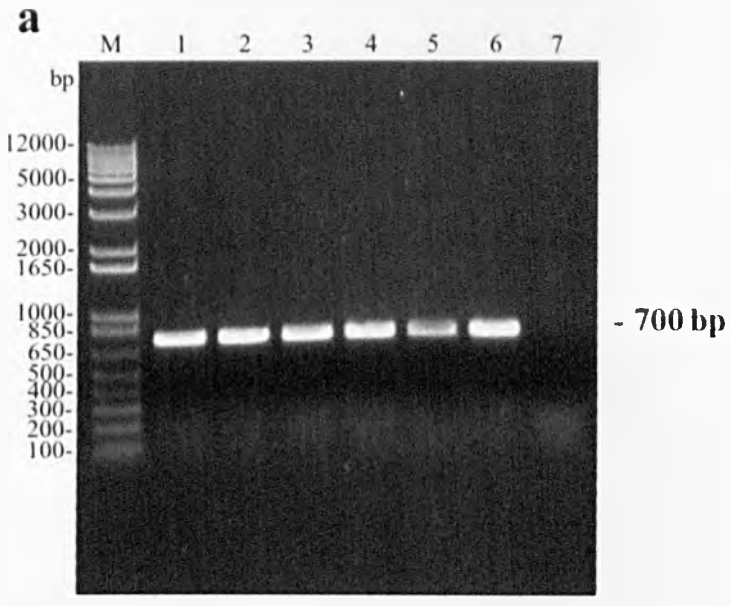
             .

pa4975       -
FWD          -
REV          C
```

APPENDIX II

Screening of *E. coli* JM109ES cultures cloned with putative *P. aeruginosa* PAO1 azoreductase genes.

Agarose gels showing the amplification of the putative *P. aeruginosa* PAO1 azoreductase gene *pa2280* (**a**), *pa2580* (**b**) and *pa4975* (**c**)contained in pET28b+ vector and cloned into *E. coli* JM109ES cells. A single band or approximately the correct size was obtained for each clone (lane 1-6) and no PCR product was obtained for negative controls (empty plasmid, lanes 7). Colony PCR was performed as described in section 2.4.8 and samples were analysed as described in section 2.3.3.



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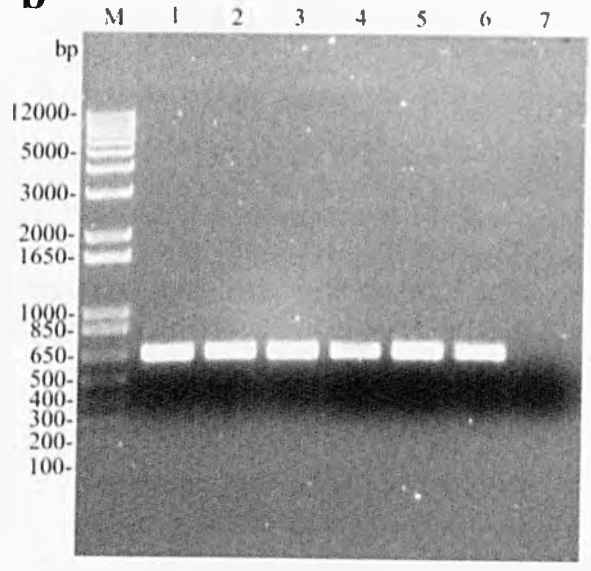
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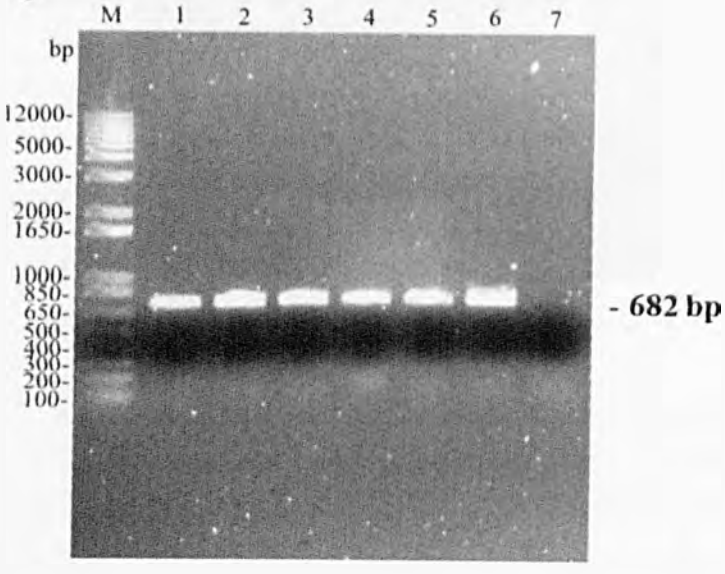
bp
12000-
5000-
3000-
2000-
1650-
1000-
850-
650-
500-
400-
300-
200-
100-
- 595 bp

c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bp
12000-
5000-
3000-
2000-
1650-
1000-
850-
650-
500-
400-
300-
200-
100-
- 682 bp

APPENDIX III

Sequence alignment of the cloned gene pa2580

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             AGAATGTGCA TGCAAGGAGA TGGCGCCCAA CAGTCCCCCG GCCACGGGGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    55         65         75         85         95
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             CTGCCACCAT ACCCACGCCG AAACAAGCGC TCATGAGCCC GAAGTGGCGA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   105        115        125        135        145
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             GCCCGATCTT CCCCATCGGT GATGTCGGCG ATATAGGCGC CAGCAACCGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   155        165        175        185        195
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             ACCTGTGGCG CCGGTGATGC CGGCCACGAT GCGTCCGGCG TAGAGGATCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   205        215        225        235        245
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   255        265        275        285        295
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             CGGATAACAA TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             ATATACCATG GGCAGCAGCC ATCATCATCA TCATCACAGC AGCGGCCTGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa2580          ---------- -------ATG AAAAACATTC TCCTGCTCAA CGGCGGAAAG
FWD             ---------- ---------- ---------- ---------- ----------
REV             TGCCGCGCGG CAGCCATATG AAAAACATTC TCCTGCTCAA CGGCGGAAAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa2580          CGTTTCGCCC ATTCCGACGG TCGCCTCAAC CAGACCCTGC ACGAAACCGC
FWD             ---------- ---------- --------AC CAGACCCTGC ACGAAACCGC
REV             CGTTTCGCCC ATTCCGACGG TCGCCTCAAC CAGACCCTGC ACGAAACCGC
```

```
                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   455         465         475         485         495
pa2580          CCTGGCCCAT CTGGACCGCC GTGGCTTCGA CCTGCGCCAG ACCTTCATCG
FWD             CCTGGCCCAT CTGGACCGCC GTGGCTTCGA CCTGCGCCAG ACCTTCATCG
REV             CCTGGCCCAT CTGGACCGCC GTGGCTTCGA CCTGCGCCAG ACCTTCATCG

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   505         515         525         535         545
pa2580          ACGGTGGCTA TGATATCCCG ACGGAGGTGG ACAAGTTCCT CTGGGCCGAC
FWD             ACGGTGGCTA TGATATCCCG ACGGAGGTGG ACAAGTTCCT CTGGGCCGAC
REV             ACGGTGGCTA TGATATCCCG ACGGAGGTGG ACAAGTTCCT CTGGGCCGAC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   555         565         575         585         595
pa2580          GTGGTGATCT ACCAGATGCC CGGCTGGTGG ATGGGCGCCC CGTGGACAGT
FWD             GTGGTGATCT ACCAGATGCC CGGCTGGTGG ATGGGCGCCC CGTGGACAGT
REV             GTGGTGATCT ACCAGATGCC CGGCTGGTGG ATGGGCGCCC CGTGGACAGT

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   605         615         625         635         645
pa2580          GAAGCGCTAC ATCGACGAAG TCTTCACGGC CGGACACGGC AGCCTCTATG
FWD             GAAGCGCTAC ATCGACGAAG TCTTCACGGC CGGACACGGC AGCCTCTATG
REV             GAAGCGCTAC ATCGACGAAG TCTTCACGGC CGGACACGGC AGCCTCTATG

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   655         665         675         685         695
pa2580          CCAACGACGG TCGTACCCGC TCCGACAGCA CGCAGAAATA TGGCAGCGGC
FWD             CCAACGACGG TCGTACCCGC TCCGACAGCA CGCAGAAATA TGGCAGCGGC
REV             CCAACGACGG TCGTACCCGC TCCGACAGCA CGCAGAAATA TGGCAGCGGC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   705         715         725         735         745
pa2580          GGTCTGGTGC AGGGCAAGCG CTACATGATC TCGGCGACCT GGAATGCACC
FWD             GGTCTGGTGC AGGGCAAGCG CTACATGATC TCGGCGACCT GGAATGCACC
REV             GGTCTGGTGC AGGGCAAGCG CTACATGATC TCGGCGACCT GGAATGCACC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   755         765         775         785         795
pa2580          ACGGCAGGCG TTCGACGATC CGAGCGACTT CTTCGAAGGA AAAGGCGTGG
FWD             ACGGCAGGCG TTCGACGATC CGAGCGACTT CTTCGAAGGA AAAGGCGTGG
REV             ACGGCAGGCG TTCGACGATC CGAGCGACTT CTTCGAAGGA AAAGGCGTGG

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   805         815         825         835         845
pa2580          ATGCGGTGTA TTTCCCCTTC CACAAGGCCA ACCAGTTCCT CGGCATGTCC
FWD             ATGCGGTGTA TTTCCCCTTC CACAAGGCCA ACCAGTTCCT CGGCATGTCC
REV             ATGCGGTGTA TTTCCCCTTC CACAAGGCCA ACCAGTTCCT CGGCATGTCC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   855         865         875         885         895
pa2580          GGCCTGCCGA CGTTCCTCGC GGTGGATGTG ATGAAGCGCC CGGACGTCCC
FWD             GGCCTGCCGA CGTTCCTCGC GGTGGATGTG ATGAAGCGCC CGGACGTCCC
REV             GGCCTGCCGA CGTTCCTCGC GGTGGATGTG ATGAAGCGCC CGGACGTCCC

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   905         915         925         935         945
pa2580          GGCTACCGTC GCTGCCTACC AGGCGCACCT GGACAGGGTG TTCGGGCGCG
FWD             GGCTACCGTC GCTGCCTACC AGGCGCACCT GGACAGGGTG TTCGGGCGCG
REV             GGCTACCGTC GCTGCCTACC AGGCGCACCT GGACAGGGTG TTCGGGCGCG
```

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                   955        965        975        985        995
pa2580          CCGGCTGA-- ---------- ---------- ---------- ----------
FWD             CCGGCTGAGT TCGAGCTCCG TCGACAAGCT TGCGGCCGCA CTCGAGCACC
REV             CCGGCTGAGT TCGAGCTCCG TCGACAAGCT -GCGGCCGCA CTCGAGCACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   1005       1015       1025       1035       1045
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             ACCACCACCA CCACTGAGAT CCGGCTGCTA ACAAAGCCCG AAAGGAAGCT
REV             AC-------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   1055       1065       1075       1085       1095
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             GAGTTGGCTG CTGCCACCGC TGAGCAATAA CTAGCATAAC CCCTTGGGGC
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   1105       1115       1125       1135       1145
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             CTCTAAACGG GTCTTGAGGG GTTTTTTGCT GAAAGGAGGA ACTATATCCG
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   1155       1165       1175       1185       1195
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             GATTGGCGAA TGGGACGCGC CCTGTAGCGG CGCATTAAGC GCGGCGGGTG
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   1205       1215       1225       1235       1245
pa2580          ---------- ---------- ---------- ---------- ----------
FWD             TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC CCTAGCGCCC
REV             ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|
                   1255       1265       1275
pa2580          ---------- ---------- -----
FWD             GCTCCCTTTC GCTTTCTTCC CTTTC
REV             ---------- ---------- -----
```

Sequence alignment of the cloned gene pa2280

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                    5          15         25         35         45
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             CACTGTGGCG CCGGTGATGC CGGCCACGAT GCGTCCGGCG TAGAGGATCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    55         65         75         85         95
pa2280          ---------- ---------- ---------- ---------- ----------
FWD             ---------- ---------- ---------- ---------- ----------
REV             AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG GAATTGTGAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                105        115        125        135        145
pa2280     ---------- ---------- ---------- ---------- ----------
FWD        ---------- ---------- ---------- ---------- ----------
REV        CGGATAACAA TTCCCCTCTA GAAATAATTT TGTTTAACTT TAAGAAGGAG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              155        165        175        185        195
pa2280     ---------- ---------- ---------- ---------- ----------
FWD        ---------- ---------- ---------- ---------- ----------
REV        ATATACCATG GGCAGCAGCC ATCATCATCA TCATCACAGC AGCGGCCTGG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              205        215        225        235        245
pa2280     ---------- -------ATG TCCGAACAAC TACCCAACCT CGATCCCGCG
FWD        ---------- CAGCCATATG TCCGAACAAC TACCCAACCT CGATCCCGCG
REV        TGCCGCGCGG CAGCCATATG TCCGAACAAC TACCCAACCT CGATCCCGCG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              255        265        275        285        295
pa2280     CTGCTCGGCG ACCCGCCCCC CGTCTCCGGG CACAGGCCGC GCATCCTCCT
FWD        CTGCTCGGCG ACCCGCCCCC CGTCTCCGGG CACAGGCCGC GCATCCTCCT
REV        CTGCTCGGCG ACCCGCCCCC CGTCTCCGGG CACAGGCCGC GCATCCTCCT

           ....|....| ....|....| ....|....| ....|....| ....|....|
              305        315        325        335        345
pa2280     GCTCTACGGC TCGACCCGCG AGCGCTCCTT CAGCCGCCTG CTGGTGCTGG
FWD        GCTCTACGGC TCGACCCGCG AGCGCTCCTT CAGCCGCCTG CTGGTGCTGG
REV        GCTCTACGGC TCGACCCGCG AGCGCTCCTT CAGCCGCCTG CTGGTGCTGG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              355        365        375        385        395
pa2280     AGGCCGCACG CCTGCTCGAA CGCTTCGGTG CCGAAACGCG GATTTTCGAC
FWD        AGGCCGCACG CCTGCTCGAA CGCTTCGGTG CCGAAACGCG GATTTTCGAC
REV        AGGCCGCACG CCTGCTCGAA CGCTTCGGTG CCGAAACGCG GATTTTCGAC

           ....|....| ....|....| ....|....| ....|....| ....|....|
              405        415        425        435        445
pa2280     CCTTCCGGGC TGCCATTGCC CGATGATGCA CCGGTGGAGC ATCCCAAGGT
FWD        CCTTCCGGGC TGCCATTGCC CGATGATGCA CCGGTGGAGC ATCCCAAGGT
REV        CCTTCCGGGC TGCCATTGCC CGATGATGCA CCGGTGGAGC ATCCCAAGGT

           ....|....| ....|....| ....|....| ....|....| ....|....|
              455        465        475        485        495
pa2280     CCGCGAGTTG CGCGACCTGG TGCAGTGGTC GGAAGGCCAG GTCTGGTGCT
FWD        CCGCGAGTTG CGCGACCTGG TGCAGTGGTC GGAAGGCCAG GTCTGGTGCT
REV        CCGCGAGTTG CGCGACCTGG TGCAGTGGTC GGAAGGCCAG GTCTGGTGCT

           ....|....| ....|....| ....|....| ....|....| ....|....|
              505        515        525        535        545
pa2280     CGCCCGAGCG CCACGGTGCG CTGTCCGCGG TATTCAAGGC GCAGATCGAC
FWD        CGCCCGAGCG CCACGGTGCG CTGTCCGCGG TATTCAAGGC GCAGATCGAC
REV        CGCCCGAGCG CCACGGTGCG CTGTCCGCGG TATTCAAGGC GCAGATCGAC

           ....|....| ....|....| ....|....| ....|....| ....|....|
              555        565        575        585        595
pa2280     TGGATTCCCC TGGCACTCGG GGCGGTGCGC CCGACCCAGG GCAAGACCCT
FWD        TGGATTCCCC TGGCACTCGG GGCGGTGCGC CCGACCCAGG GCAAGACCCT
REV        TGGATTCCCC TGGCACTCGG GGCGGTGCGC CCGACCCAGG GCAAGACCCT

           ....|....| ....|....| ....|....| ....|....| ....|....|
```

```
                605         615         625         635         645
pa2280      GGCGCTGATG CAGGTCTGCG GCGGCTCGCA GTCGTTCAAC GTGGTCAACC
FWD         GGCGCTGATG CAGGTCTGCG GCGGCTCGCA GTCGTTCAAC GTGGTCAACC
REV         GGCGCTGATG CAGGTCTGCG GCGGCTCGCA GTCGTTCAAC GTGGTCAACC

            ....|....| ....|....| ....|....| ....|....| ....|....|
                655         665         675         685         695
pa2280      AGTTGCGCGT GCTGGGTCGC TGGATGCGCA TGTTCACCAT CCCCAACCAG
FWD         AGTTGCGCGT GCTGGGTCGC TGGATGCGCA TGTTCACCAT CCCCAACCAG
REV         AGTTGCGCGT GCTGGGTCGC TGGATGCGCA TGTTCACCAT CCCCAACCAG

            ....|....| ....|....| ....|....| ....|....| ....|....|
                705         715         725         735         745
pa2280      TCCTCGGTTC CCAAGGCCTA CCTGGAGTTC GACGAAGCGG GCCGGATGAA
FWD         TCCTCGGTTC CCAAGGCCTA CCTGGAGTTC GACGAAGCGG GCCGGATGAA
REV         TCCTCGGTTC CCAAGGCCTA CCTGGAGTTC GACGAAGCGG GCCGGATGAA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                755         765         775         785         795
pa2280      GCCATCGCCC TACTACGACC GGGTGGTCGA CGTGATGGAG GAGTTGTTCA
FWD         GCCATCGCCC TACTACGACC GGGTGGTCGA CGTGATGGAG GAGTTGTTCA
REV         GCCATCGCCC TACTACGACC GGGTGGTCGA CGTGATGGAG GAGTTGTTCA

            ....|....| ....|....| ....|....| ....|....| ....|....|
                805         815         825         835         845
pa2280      AGTTCACCCT GCTCCTGCGC GAGCGCACGG ACTTCCTGGT GGACCGCTAT
FWD         AGTTCACCCT GCTCCTGCGC GAGCGCACGG ACTTCCTGGT GGACCGCTAT
REV         AGTTCACCCT GCTCCTGCGC GAGCGCACGG ACTTCCTGGT GGACCGCTAT

            ....|....| ....|....| ....|....| ....|....| ....|....|
                855         865         875         885         895
pa2280      TCGGAGCGCA AGGAAAGCGC CGAGCAGCTT TCCGCGCGGG TCGACCAGCG
FWD         TCGGAGCGCA AGGAAAGCGC CGAGCAGCTT TCCGCGCGGG TCGACCAGCG
REV         TCGGAGCGCA AGGAAAGCGC CGAGCAGCTT TCCGCGCGGG TCGACCAGCG

            ....|....| ....|....| ....|....| ....|....| ....|....|
                905         915         925         935         945
pa2280      TTCGCTCTGA ---------- ---------- ---------- ----------
FWD         TTCGCTCTGA GCTCCGTCGA CAAGCTTGCG GCCGCACTCG AGCACCACCA
REV         TTCGCTCTGA GCTCCGTCGA CAAGCTTGCG GCCGCACTCG AGCA------

            ....|....| ....|....| ....|....| ....|....| ....|....|
                955         965         975         985         995
pa2280      ---------- ---------- ---------- ---------- ----------
FWD         CCACCACCAC TGAGATCCGG CTGCTAACAA AGCCCGAAAG GAAGCTGAGT
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
               1005        1015        1025        1035        1045
pa2280      ---------- ---------- ---------- ---------- ----------
FWD         TGGCTGCTGC CACCGCTGAG CAATAACTAG CATAACCCCT TGGGGCCTCT
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
               1055        1065        1075        1085        1095
pa2280      ---------- ---------- ---------- ---------- ----------
FWD         AAACGGGTCT TGAGGGGTTT TTTGCTGAAA GGAGGAACTA TATCCGGATT
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
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```
                1105       1115       1125       1135       1145
pa2280     ---------- ---------- ---------- ---------- ----------
FWD        GGCGAATGGG ACGCGCCCTG TAGCGGCGCA TTAAGCGCGG CGGGTGTGGT
REV        ---------- ---------- ---------- ---------- ----------

           ....|....| ....|....| ....|....| ....
              1155       1165       1175
pa2280     ---------- ---------- ---------- ---.
FWD        GGTTACGCGC AGCGTGACCG CTACACTTGC CAG.
REV        ---------- ---------- ---------- ---.
```

Sequence alignment of the cloned gene pa1204

```
           ....|....| ....|....| ....|....| ....|....| ....|....|
              5          15         25         35         45
pa1204     ---------- ---------- ---------- ---------- ----------
FWD        ---------- ---------- ---------- ---------- ----------
REV        CGCACCTGTG GCGCCCGGTG ATGCCGGGCC ACGATGCGTC CGGCGTAGAG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              55         65         75         85         95
pa1204     ---------- ---------- ---------- ---------- ----------
FWD        ---------- ---------- ---------- ---------- ----------
REV        GATCGAGATC TCGATCCCGC GAAATTAATA CGACTCACTA TAGGGGAATT

           ....|....| ....|....| ....|....| ....|....| ....|....|
              105        115        125        135        145
pa1204     ---------- ---------- ---------- ---------- ----------
FWD        ---------- ---------- ---------- ---------- ----------
REV        GTGAGCGGAT AACAATTCCC CTCTAGAAAT AATTTTGTTT AACTTTAAGA

           ....|....| ....|....| ....|....| ....|....| ....|....|
              155        165        175        185        195
pa1204     ---------- ---------- ---------- ---------- ----------
FWD        ---------- ---------- ---------- ---------- ----------
REV        AGGAGATATA CCATGGGCAG CAGCCATCAT CATCATCATC ACAGCAGCGG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              205        215        225        235        245
pa1204     ---------- ---------- --ATGAGCGA CGACATCAAG GTATTGGGCA
FWD        ---------- CGCGGCAGCC ATATGAGCGA CGACATCAAG GTATTGGGCA
REV        CCTGGTGCCG CGCGGCAGCC ATATGAGCGA CGACATCAAG GTATTGGGCA

           ....|....| ....|....| ....|....| ....|....| ....|....|
              255        265        275        285        295
pa1204     TTTCCGGCAG CCTGCGCAGC GGCTCCTACA ACAGCGCGGC GCTGCAGGAG
FWD        TTTCCGGCAG CCTGCGCAGC GGCTCCTACA ACAGCGCGGC GCTGCAGGAG
REV        TTTCCGGCAG CCTGCGCAGC GGCTCCTACA ACAGCGCGGC GCTGCAGGAG

           ....|....| ....|....| ....|....| ....|....| ....|....|
              305        315        325        335        345
pa1204     GCGATTGGCC TGGTCCCGCC GGGCATGAGC ATCGAGCTGG CGGACATCTC
FWD        GCGATTGGCC TGGTCCCGCC GGGCATGAGC ATCGAGCTGG CGGACATCTC
REV        GCGATTGGCC TGGTCCCGCC GGGCATGAGC ATCGAGCTGG CGGACATCTC

           ....|....| ....|....| ....|....| ....|....| ....|....|
              355        365        375        385        395
```

```
pa1204      CGGCATCCCG CTGTACAACG AGGACGTCTA CGCCCTCGGC TTCCCGCCCG
FWD         CGGCATCCCG CTGTACAACG AGGACGTCTA CGCCCTCGGC TTCCCGCCCG
REV         CGGCATCCCG CTGTACAACG AGGACGTCTA CGCCCTCGGC TTCCCGCCCG

            ....|....| ....|....| ....|....| ....|....| ....|....|
               405        415        425        435        445
pa1204      CGGTGGAACG CTTCCGCGAG CAGATTCGCG CGGCGGACGC GCTGCTGTTC
FWD         CGGTGGAACG CTTCCGCGAG CAGATTCGCG CGGCGGACGC GCTGCTGTTC
REV         CGGTGGAACG CTTCCGCGAG CAGATTCGCG CGGCGGACGC GCTGCTGTTC

            ....|....| ....|....| ....|....| ....|....| ....|....|
               455        465        475        485        495
pa1204      GCCACCCCGG AATACAACTA TTCGATGGCC GGGGTGCTGA AGAATGCCAT
FWD         GCCACCCCGG AATACAACTA TTCGATGGCC GGGGTGCTGA AGAATGCCAT
REV         GCCACCCCGG AATACAACTA TTCGATGGCC GGGGTGCTGA AGAATGCCAT

            ....|....| ....|....| ....|....| ....|....| ....|....|
               505        515        525        535        545
pa1204      CGACTGGGCC TCGCGGCCGC CGGAGCAGCC GTTCTCCGGC AAGCCGGCGG
FWD         CGACTGGGCC TCGCGGCCGC CGGAGCAGCC GTTCTCCGGC AAGCCGGCGG
REV         CGACTGGGCC TCGCGGCCGC CGGAGCAGCC GTTCTCCGGC AAGCCGGCGG

            ....|....| ....|....| ....|....| ....|....| ....|....|
               555        565        575        585        595
pa1204      CGATCCTCGG CGCCAGCGCC GGGCGTTTCG GCACCGCGCG GGCGCAGTAT
FWD         CGATCCTCGG CGCCAGCGCC GGGCGTTTCG GCACCGCGCG GGCGCAGTAT
REV         CGATCCTCGG CGCCAGCGCC GGGCGTTTCG GCACCGCGCG GGCGCAGTAT

            ....|....| ....|....| ....|....| ....|....| ....|....|
               605        615        625        635        645
pa1204      CACTTGCGCC AGACGCTGGT GTTCCTCGAC GTTCATCCGC TGAACAAGCC
FWD         CACTTGCGCC AGACGCTGGT GTTCCTCGAC GTTCATCCGC TGAACAAGCC
REV         CACTTGCGCC AGACGCTGGT GTTCCTCGAC GTTCATCCGC TGAACAAGCC

            ....|....| ....|....| ....|....| ....|....| ....|....|
               655        665        675        685        695
pa1204      GGAAGTGATG ATCTCCAGCG CGCAGAACGC CTTCGATGCC CAGGGCCGGC
FWD         GGAAGTGATG ATCTCCAGCG CGCAGAACGC CTTCGATGCC CAGGGCCGGC
REV         GGAAGTGATG ATCTCCAGCG CGCAGAACGC CTTCGATGCC CAGGGCCGGC

            ....|....| ....|....| ....|....| ....|....| ....|....|
               705        715        725        735        745
pa1204      TGCTCGACGA CAAGGCGCGC GAGCTGATCC AGCAGCAGTT GCAGGCCCTG
FWD         TGCTCGACGA CAAGGCGCGC GAGCTGATCC AGCAGCAGTT GCAGGCCCTG
REV         TGCTCGACGA CAAGGCGCGC GAGCTGATCC AGCAGCAGTT GCAGGCCCTG

            ....|....| ....|....| ....|....| ....|....| ....|....|
               755        765        775        785        795
pa1204      CAGCTATGGG TGCGCCGCCT GCGCGGTTGA ---------- ----------
FWD         CAGCTATGGG TGCGCCGCCT GCGCGGTTGA ATGCCTCGTC ACCGGAGAGC
REV         CAGCTATGGG TGCGCCGCCT GCGCGGTTGA ATGCCTCGTC ACCGGAGAGC

            ....|....| ....|....| ....|....| ....|....| ....|....|
               805        815        825        835        845
pa1204      ---------- ---------- ---------- ---------- ----------
FWD         TCCGTCGACA AGCTTGCGGC CGCACTCGAG CACCACCACC ACCACCACTG
REV         TCCGTCGACA AGCTTGCGGC CGCACT---- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
               855        865        875        885        895
```

```
pa1204      ---------- ---------- ---------- ---------- ----------
FWD         AGATCCGGCT GCTAACAAAG CCCGAAAGGA AGCTGAGTTG GCTGCTGCCA
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....|
               905        915        925        935        945
pa1204      ---------- ---------- ---------- ---------- ----------
FWD         CCGCTGAGCA ATAACTAGCA TAACCCCTTG GGGCCCTCTA AACGGGTCTT
REV         ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....
               955        965        975        985
pa1204      ---------- ---------- ---------- ---------- ----
FWD         GAGGGGTTTT TTGCTGAAAG GAGGAACTAT ATCCGGGATT GGCG
REV         ---------- ---------- ---------- ---------- ----
```

Sequence alignment of the cloned gene pa0949

```
            ....|....| ....|....| ....|....| ....|....| ....|....|
               5          15         25         35         45
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         ---------- ---------- ---------- ---------- ----------
REV         GCCGGCCACG ATGCGTCCCG GCGTAGAGGA TCGAGATCTC GATCCCGCGA

            ....|....| ....|....| ....|....| ....|....| ....|....|
               55         65         75         85         95
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         ---------- ---------- ---------- ---------- ----------
REV         AATTAATACG ACTCACTATA GGGGAATTGT GAGCGGATAA CAATTCCCCT

            ....|....| ....|....| ....|....| ....|....| ....|....|
               105        115        125        135        145
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         ---------- ---------- ---------- ---------- ----------
REV         CTAGAAATAA TTTTGTTTAA CTTTAAGAAG GAGATATACC ATGGGCAGCA

            ....|....| ....|....| ....|....| ....|....| ....|....|
               155        165        175        185        195
pa0949      ---------- ---------- ---------- ---------- ----------
FWD         ---------- ---------- ---------- ---------- ----------
REV         GCCATCATCA TCATCATCAC AGCAGCGGCC TGGTGCCGCG CGGCAGCCAT

            ....|....| ....|....| ....|....| ....|....| ....|....|
               205        215        225        235        245
pa0949      ATGAGCAGTC CCTACATCCT GGTGCTGTAC TACAGTCGCC ATGGCGCTAC
FWD         ATGAGCAGTC CCTACATCCT GGTGCTGTAC TACAGTCGCC ATGGCGCTAC
REV         ATGAGCAGTC CCTACATCCT GGTGCTGTAC TACAGTCGCC ATGGCGCTAC

            ....|....| ....|....| ....|....| ....|....| ....|....|
               255        265        275        285        295
pa0949      CGCGGAAATG GCCCGGCAGA TCGCCCGTGG CGTCGAACAG GGCGGCTTCG
FWD         CGCGGAAATG GCCCGGCAGA TCGCCCGTGG CGTCGAACAG GGCGGCTTCG
REV         CGCGGAAATG GCCCGGCAGA TCGCCCGTGG CGTCGAACAG GGCGGCTTCG

            ....|....| ....|....| ....|....| ....|....| ....|....|
               305        315        325        335        345
pa0949      AGGCGCGTGT ACGCACGGTT CCCGCGGTAT CCACCGAATG CGAAGCGGTC
```

```
FWD          AGGCGCGTGT ACGCACGGTT CCCGCGGTAT CCACCGAATG CGAAGCGGTC
REV          AGGCGCGTGT ACGCACGGTT CCCGCGGTAT CCACCGAATG CGAAGCGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa0949       GCCCCCGACA TCCCCGCGGA GGGGGCGCTG TACGCAACCC TGGAGGACCT
FWD          GCCCCCGACA TCCCCGCGGA GGGGGCGCTG TACGCAACCC TGGAGGACCT
REV          GCCCCCGACA TCCCCGCGGA GGGGGCGCTG TACGCAACCC TGGAGGACCT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa0949       GAAGAACTGC GCGGGCCTGG CCCTCGGCAG CCCGACCCGC TTCGGCAACA
FWD          GAAGAACTGC GCGGGCCTGG CCCTCGGCAG CCCGACCCGC TTCGGCAACA
REV          GAAGAACTGC GCGGGCCTGG CCCTCGGCAG CCCGACCCGC TTCGGCAACA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
pa0949       TGGCTTCCCC GCTGAAATAC TTCCTCGACG GTACCAGCAG CCTGTGGCTG
FWD          TGGCTTCCCC GCTGAAATAC TTCCTCGACG GTACCAGCAG CCTGTGGCTG
REV          TGGCTTCCCC GCTGAAATAC TTCCTCGACG GTACCAGCAG CCTGTGGCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
pa0949       ACCGGCAGCC TGGTCGGCAA GCCGGCAGCG GTCTTCACCT CCACCGCCAG
FWD          ACCGGCAGCC TGGTCGGCAA GCCGGCAGCG GTCTTCACCT CCACCGCCAG
REV          ACCGGCAGCC TGGTCGGCAA GCCGGCAGCG GTCTTCACCT CCACCGCCAG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
pa0949       CCTGCACGGC GGCCAGGAGA CCACTCAGTT ATCGATGCTG TTGCCATTGC
FWD          CCTGCACGGC GGCCAGGAGA CCACTCAGTT ATCGATGCTG TTGCCATTGC
REV          CCTGCACGGC GGCCAGGAGA CCACTCAGTT ATCGATGCTG TTGCCATTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
pa0949       TGCACCACGG CATGCTGGTC CTGGGCATTC CCTACAGCGA ACCCGCCCTG
FWD          TGCACCACGG CATGCTGGTC CTGGGCATTC CCTACAGCGA ACCCGCCCTG
REV          TGCACCACGG CATGCTGGTC CTGGGCATTC CCTACAGCGA ACCCGCCCTG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   655        665        675        685        695
pa0949       CTGGAAACCC GCGGCGGCGG CACGCCTTAC GGCGCCAGCC ACTTCGCCGG
FWD          CTGGAAACCC GCGGCGGCGG CACGCCTTAC GGCGCCAGCC ACTTCGCCGG
REV          CTGGAAACCC GCGGCGGCGG CACGCCTTAC GGCGCCAGCC ACTTCGCCGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   705        715        725        735        745
pa0949       CGCCGATGGC AAGCGCAGCC TCGATGAGCA CGAACTGACC CTGTGTCGCG
FWD          CGCCGATGGC AAGCGCAGCC TCGATGAGCA CGAACTGACC CTGTGTCGCG
REV          CGCCGATGGC AAGCGCAGCC TCGATGAGCA CGAACTGACC CTGTGTCGCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   755        765        775        785        795
pa0949       CGCTGGGCAA ACGCCTGGCG GAAACCGCCG GCAAGCTGGG GAGTTGA---
FWD          CGCTGGGCAA ACGCCTGGCG GAAACCGCCG GCAAGCTGGG GAGTTGAAAT
REV          CGCTGGGCAA ACGCCTGGCG GAAACCGCCG GCAAGCTGGG GAGTTGAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   805        815        825        835        845
pa0949       ---------- ---------- ---------- ---------- ----------
```

```
FWD          GGCCCGCAAG AGCTCCGTCG ACAAGCTTGC GGCCGCACTC GAGCACCACC
REV          GGCCCGCAAG AGCTCCGTCG ACAAGCTTGC GGCCGCACTC GAGCA-----

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   855        865        875        885        895
pa0949       ---------- ---------- ---------- ---------- ----------
FWD          ACCACCACCA CTGAGATCCG GCTGCTAACA AAGCCCGAAA GGAAGCTGAG
REV          ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   905        915        925        935        945
pa0949       ---------- ---------- ---------- ---------- ----------
FWD          TTGGCTGCTG CCACCGCTGA GCAATAACTA GCATAACCCC TTGGGGCCTC
REV          ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   955        965        975        985        995
pa0949       ---------- ---------- ---------- ---------- ----------
FWD          TAAACGGGTC TTGAGGGGTT TTTTGCTGAA AGGAGGAACT ATATCCGGAT
REV          ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|
                  1005       1015       1025       1035       1045
pa0949       ---------- ---------- ---------- ---------- -----
FWD          TGGCGAATGG GACGCGCCCT GTAGCGGCGC ATTAAGCGCG CGGTT
REV          ---------- ---------- ---------- ---------- -----
```

Sequence alignment of the cloned gene pa4975

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          TGATGTCGGC GATATAGGCG CCAGCAACGC ACCTGTGGCG CCGGTGATGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                     55         65         75         85         95
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          CGGCCACGAT GCGTCCGGCG TAGAGGATCG AGATCTCGAT CCCGCGAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    105        115        125        135        145
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          TAATACGACT CACTATAGGG GAATTGTGAG CGGATAACAA TTCCCCTCTA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    155        165        175        185        195
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ---------- ---------- ---------- ---------- ----------
REV          GAAATAATTT TGTTTAACTT TAAGAAGGAG ATATACCATG GGCAGCAGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                    205        215        225        235        245
pa4975       ---------- ---------- ---------- ---------- -------ATG
FWD          ---------- ---------- ---------- --------CG GCAGCATATG
```

```
REV          ATCATCATCA TCATCACAGC AGCGGCCTGG TGCCGCGCGG CAGCCATATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   255        265        275        285        295
pa4975       AACGTACTGA TCGTCCACGC CCACAACGAA CCGCAATCCT TCACCCGCGC
FWD          AACGTACTGA TCGTCCACGC CCACAACGAA CCGCAATCCT TCACCCGCGC
REV          AACGTACTGA TCGTCCACGC CCACAACGAA CCGCAATCCT TCACCCGCGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   305        315        325        335        345
pa4975       GCTCTGTGAC CAGGCATGCG AGACCCTGGC AGGCCAGGGC CACGCGGTGC
FWD          GCTCTGTGAC CAGGCATGCG AGACCCTGGC AGGCCAGGGC CACGCGGTGC
REV          GCTCTGTGAC CAGGCATGCG AGACCCTGGC AGGCCAGGGC CACGCGGTGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   355        365        375        385        395
pa4975       AGGTCTCGGA TCTCTACGCG ATGAACTGGA ATCCGGTGGC CAGTGCCGCC
FWD          AGGTCTCGGA TCTCTACGCG ATGAACTGGA ATCCGGTGGC CAGTGCCGCC
REV          AGGTCTCGGA TCTCTACGCG ATGAACTGGA ATCCGGTGGC CAGTGCCGCC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   405        415        425        435        445
pa4975       GACTTCGCCG AGCGCGCCGA TCCCGACTAC CTGGTGTACG CCCTGGAGCA
FWD          GACTTCGCCG AGCGCGCCGA TCCCGACTAC CTGGTGTACG CCCTGGAGCA
REV          GACTTCGCCG AGCGCGCCGA TCCCGACTAC CTGGTGTACG CCCTGGAGCA

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   455        465        475        485        495
pa4975       GCGCGAGAGC GTCAAGCGCC AGAGCCTGGC CGCCGACATC CAGGCCGAGC
FWD          GCGCGAGAGC GTCAAGCGCC AGAGCCTGGC CGCCGACATC CAGGCCGAGC
REV          GCGCGAGAGC GTCAAGCGCC AGAGCCTGGC CGCCGACATC CAGGCCGAGC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   505        515        525        535        545
pa4975       TGGACAAGCT GCTGTGGGCC GACCTGCTGA TCCTCAACTT TCCGATCTAC
FWD          TGGACAAGCT GCTGTGGGCC GACCTGCTGA TCCTCAACTT TCCGATCTAC
REV          TGGACAAGCT GCTGTGGGCC GACCTGCTGA TCCTCAACTT TCCGATCTAC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   555        565        575        585        595
pa4975       TGGTTCTCGG TGCCGGCGAT CCTCAAGGGG TGGTTCGACC GGGTACTGGT
FWD          TGGTTCTCGG TGCCGGCGAT CCTCAAGGGG TGGTTCGACC GGGTACTGGT
REV          TGGTTCTCGG TGCCGGCGAT CCTCAAGGGG TGGTTCGACC GGGTACTGGT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   605        615        625        635        645
pa4975       GTCCGGGGTC TGCTATGGCG GCAAGCGCTT CTACGACCAG GGTGGCCTGG
FWD          GTCCGGGGTC TGCTATGGCG GCAAGCGCTT CTACGACCAG GGTGGCCTGG
REV          GTCCGGGGTC TGCTATGGCG GCAAGCGCTT CTACGACCAG GGTGGCCTGG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   655        665        675        685        695
pa4975       CCGGCAAGAA GGCGCTGGTC AGCCTGACCC TGGGCGGGCG CCAGCACATG
FWD          CCGGCAAGAA GGCGCTGGTC AGCCTGACCC TGGGCGGGCG CCAGCACATG
REV          CCGGCAAGAA GGCGCTGGTC AGCCTGACCC TGGGCGGGCG CCAGCACATG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                   705        715        725        735        745
pa4975       TTCGGCGAGG GTGCCATCCA CGGACCGCTG GAGGACATGC TGCGGCCGAT
FWD          TTCGGCGAGG GTGCCATCCA CGGACCGCTG GAGGACATGC TGCGGCCGAT
```

```
REV          TTCGGCGAGG GTGCCATCCA CGGACCGCTG GAGGACATGC TGCGGCCGAT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                755        765        775        785        795
pa4975       CCTGCGCGGC ACCCTGGCCT ATGTCGGCAT GCAGGTGCTG GAGCCCTTCG
FWD          CCTGCGCGGC ACCCTGGCCT ATGTCGGCAT GCAGGTGCTG GAGCCCTTCG
REV          CCTGCGCGGC ACCCTGGCCT ATGTCGGCAT GCAGGTGCTG GAGCCCTTCG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                805        815        825        835        845
pa4975       TCGCCTGGCA CGTGCCATAC ATCAGCGAGG AAGCGCGCGG CAACTTCCTG
FWD          TCGCCTGGCA CGTGCCATAC ATCAGCGAGG AAGCGCGCGG CAACTTCCTG
REV          TCGCCTGGCA CGTGCCATAC ATCAGCGAGG AAGCGCGCGG CAACTTCCTG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                855        865        875        885        895
pa4975       CGCGCCTACC GGGCGCGGCT GGAAAATCTC GAGCAGGATG TACCCCTGCG
FWD          CGCGCCTACC GGGCGCGGCT GGAAAATCTC GAGCAGGATG TACCCCTGCG
REV          CGCGCCTACC GGGCGCGGCT GGAAAATCTC GAGCAGGATG TACCCCTGCG

             ....|....| ....|....| ....|....| ....|....| ....|....|
                905        915        925        935        945
pa4975       GTTCCCGCGG CTGGAGCAGT TCGACGCGCT GCTCCAGCCG CTGGCGCGCT
FWD          GTTCCCGCGG CTGGAGCAGT TCGACGCGCT GCTCCAGCCG CTGGCGCGCT
REV          GTTCCCGCGG CTGGAGCAGT TCGACGCGCT GCTCCAGCCG CTGGCGCGCT

             ....|....| ....|....| ....|....| ....|....| ....|....|
                955        965        975        985        995
pa4975       GA-------- ---------- ---------- ---------- ----------
FWD          GAGCTCCGTC GACAAGCTTG CGGCCGCACT CGAGCACCAC CACCACCACC
REV          GAGCTCCGTC GACAAGCTTG CGGCCGCACT ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                1005       1015       1025       1035       1045
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          ACTGAGATCC GGCTGCTAAC AAAGCCCGAA AGGAAGCTGA GTTGGCTGCT
REV          ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                1055       1065       1075       1085       1095
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          GCCACCGCTG AGCAATAACT AGCATAACCC CTTGGGGCCT CTAAACGGGT
REV          ---------- ---------- ---------- ---------- ----------

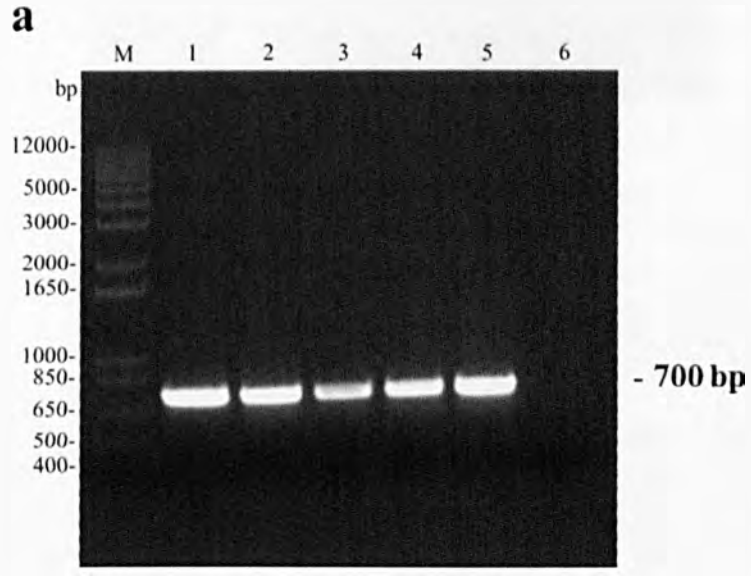
             ....|....| ....|....| ....|....| ....|....| ....|....|
                1105       1115       1125       1135       1145
pa4975       ---------- ---------- ---------- ---------- ----------
FWD          CTTGAGGGGT TTTTTGCTGA AAGGAGGAAC TATATCCGGA TTGGCGAATG
REV          ---------- ---------- ---------- ---------- ----------

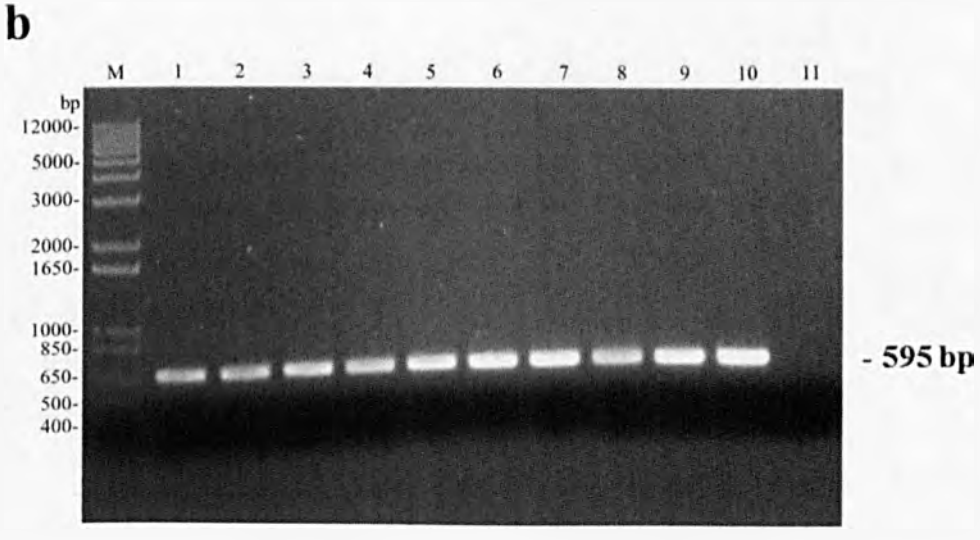
             ....|....| ....|....| ....|....| ..
                1155       1165       1175
pa4975       ---------- ---------- ---------- --
FWD          GGACGCGCCC TGTAGCGGCG CATTAAGCGC GC
REV          ---------- ---------- ---------- --
```

APPENDIX IV

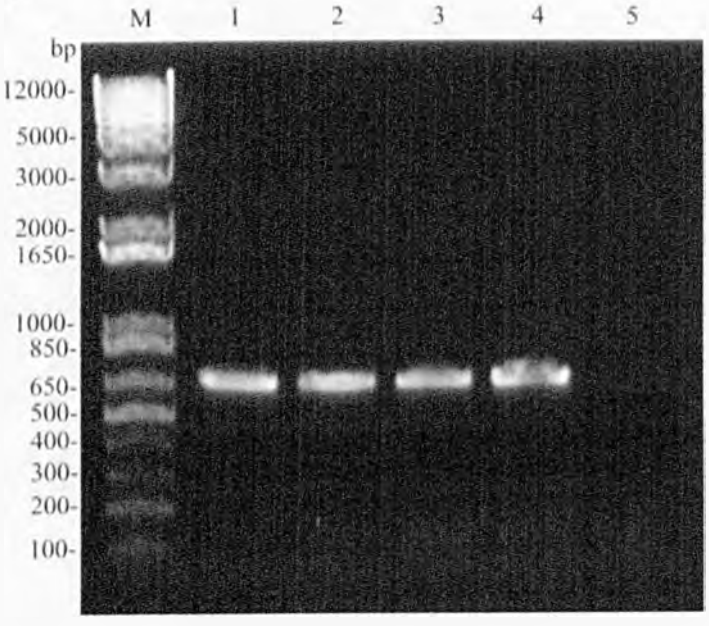
Screening of *E. coli* BL21 cultures cloned with putative *P. aeruginosa* PAO1 azoreductase genes.

Agarose gels showing the amplification of the putative *P. aeruginosa* PAO1 azoreductase gene *pa2280* (**a**), *pa2580* (**b**) and *pa4975* (**c**)contained in pET28b+ vector and cloned into *E. coli* JM109ES cells. A single band or approximately the correct size was obtained for each clone (*pa2280* lane 1-5; *pa2580* lane 1-10; *pa4975* lane 1-4) and no PCR product was obtained for negative controls (empty plasmid; *pa2280* lane 6; *pa2580* lane 11; *pa4975* lane 5). Colony PCR was performed as described in section 2.4.8 and samples were analysed as described in section 2.3.3.



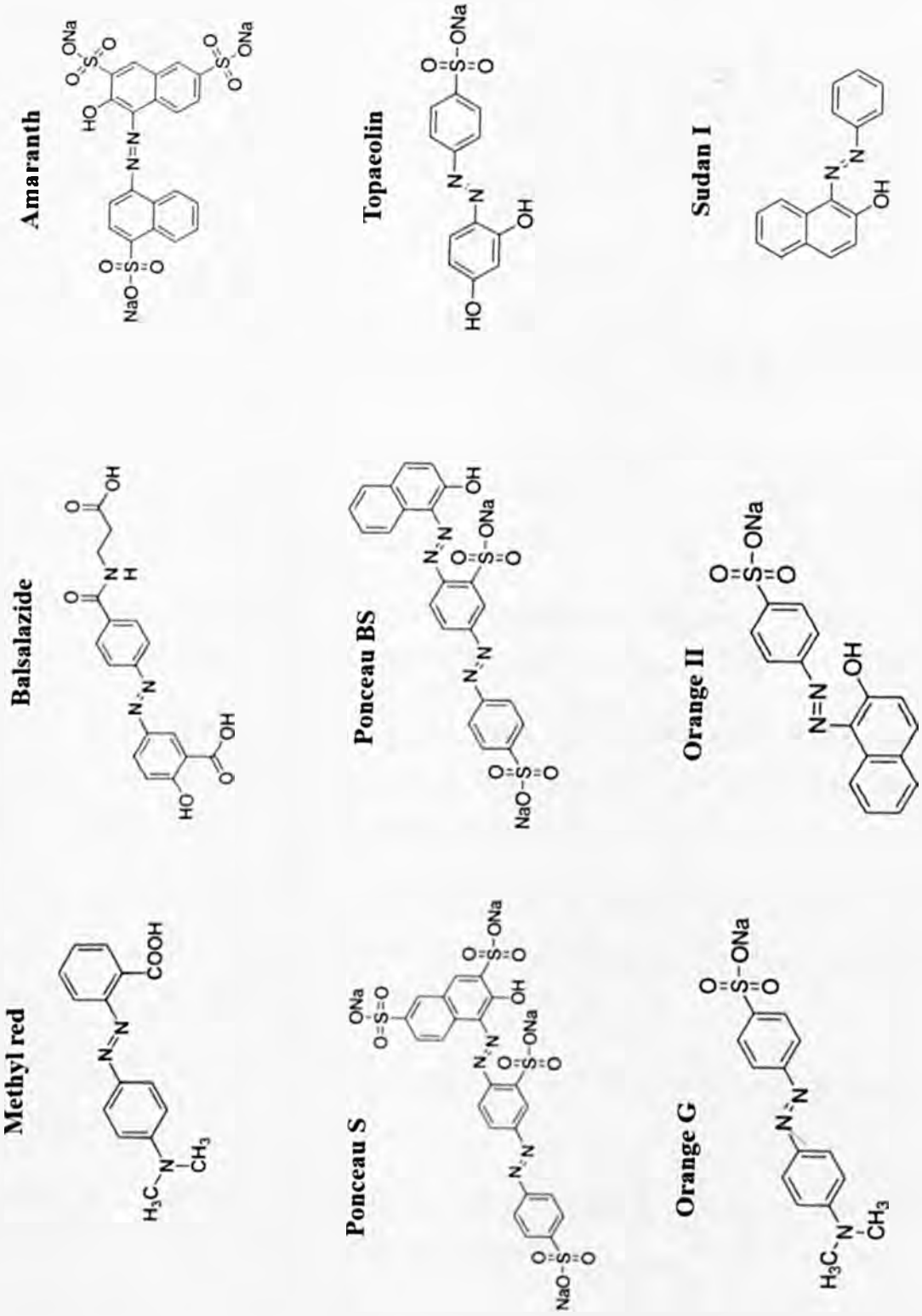


c

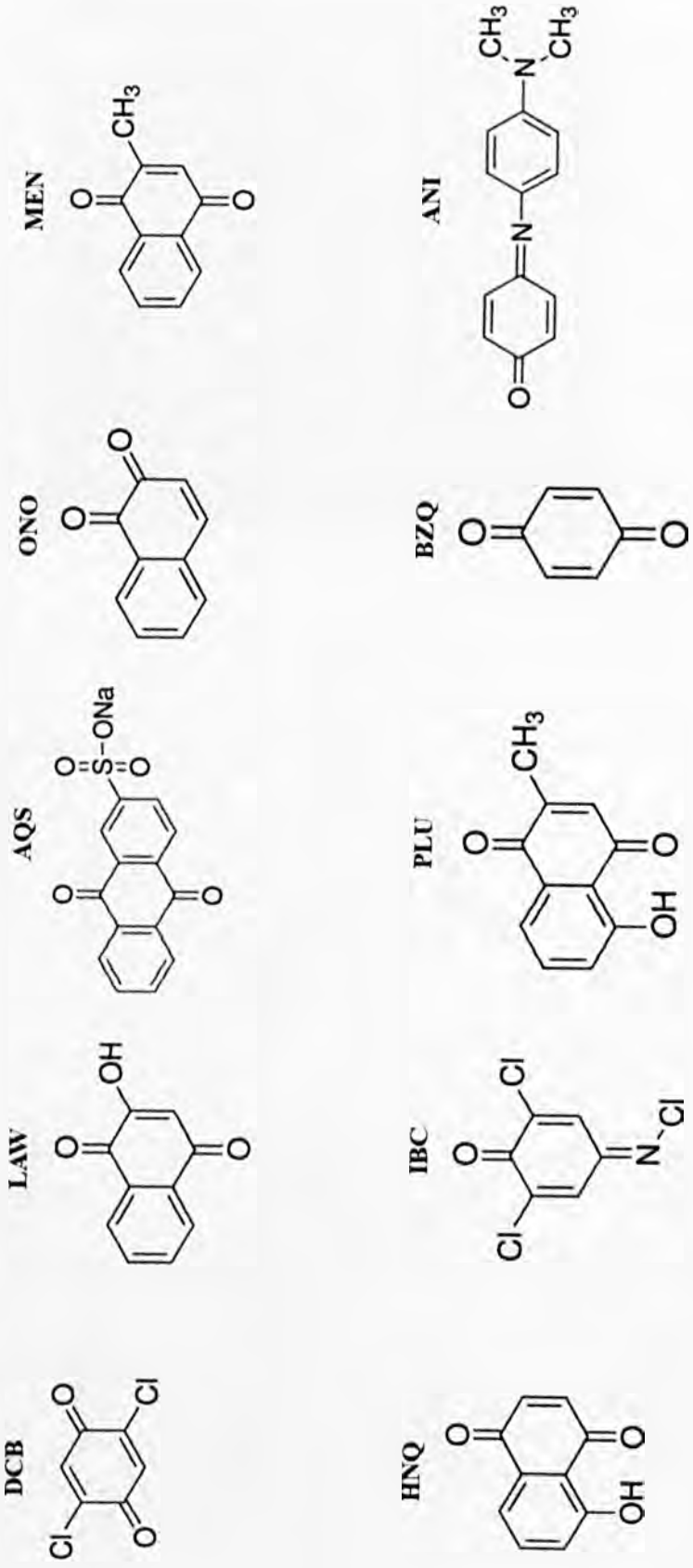

M
1
2
3
4
5
bp
12000-
5000-
3000-
2000-
1650-
1000-
850-
650-
500-
400-
300-
200-
100-
- 682 bp

APPENDIX V

Azo compound structures



Quinone structures



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