Regulation of rhamnolipid biosynthesis in the *Pseudomonas aeruginosa* PAO1 biofilm population

by

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I dedicate this manuscript in memory of my grandmother Ouma Motjie

SUMMARY

Regulation of rhamnolipid biosynthesis in the *Pseudomonas aeruginosa* PAO1 biofilm population

by

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for the degree M Sc

Pseudomonas aeruginosa a ubiquitous environmental bacterium and an opportunistic human pathogen forms biofilms through a series of interactions between the cells and adherence to surfaces Not only does rhamnolipid contribute to the pathogenic potential of *P aeruginosa* but it has also been reported that the bacterium utilises rhamnolipid to actively maintain the void spaces surrounding microcolonies thus contributing to the architecture of *P aeruginosa* biofilms The *P aeruginosa rhlAB* operon encodes the enzyme rhamnosyltransferase 1 which produces mono rhamnolipid and the induction of *rhlAB* is dependent on the quorum sensing transcription activator RhlR complexed with the autoinducer *N* butyryl homoserine lactone In this study several aspects related to rhamnolipid biosynthesis and regulation in *P aeruginosa* PAO1 were investigated

As a first step a biochemical assay was developed and optimised whereby the concentration of rhamnolipid could be accurately quantified following its extraction from small sample volumes Although the optimised rhamnolipid assay is not able to distinguish between different rhamnolipids or between different homologs of a specific rhamnolipid it is however simple to perform cost effective and does not rely on the use of specialised equipment Subsequently an *rhlAB* deficient mutant strain of *P aeruginosa* PAO1 strain was generated For this purpose three allelic exchange

strategies i e plasmid incompatibility the use of a SacB counter selectable marker and a combination of these approaches were investigated by making use of newly constructed allelic exchange vector systems The results that were obtained indicated that of the three approaches the latter was most efficient in generating the desired *P aeruginosa* mutant strain and 90% of the derived strains were found to be double reciprocal mutants

Reporter gene technology using the genes encoding for stable and unstable variants of the green fluorescent protein (GFP) was finally used to investigate the transcriptional activity of the *rhlA* promoter in *P aeruginosa* biofilms under conditions of continuous flow using glass as substratum For this purpose mini CTX GFP reporter vectors containing stable and unstable variants of the *gfp* reporter gene were constructed that allow for integration of a single copy of the transcriptional fusion in a defined non essential region onto the *P aeruginosa* genome Several global regulators have been reported to play a role in regulating quorum sensing and/or rhamnolipid biosynthesis in *P aeruginosa* amongst other the sigma factors RpoS and RpoN Therefore *rhlA* promoter activity was also investigated in biofilms of *P aeruginosa* strains lacking either RpoN or RpoS Although structural differences between the biofilms formed by the *P aeruginosa* wild type PAO1 and respective mutant strains were noted transcription of *rhlA* appeared to be constitutive from 24 h onwards and did not appear to be localised to specific areas within the microcolonies or biofilms These results combined with those obtained by batch analysis indicated that RpoS positively regulates *rhlA* transcription whilst RpoN did not appear to influence *rhlA* promoter activity under the conditions used in this study

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	Absorbance
Ap	ampicillin
Ap^R	ampicillin resistance
ATP	adenosine triphosphate
bp	base pair
C	degrees Celsius
Cb	carbenicillin
CFU	colony forming units
C_4 HSL	N butyryl L homoserine lactone
Cm	chloramphenicol
cmc	critical micelle concentration
CTAB	cetyltrimethylammonium bromide
ddH_2O	deionized distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5 triphosphate
dTDP L rhamnose	deoxy thymidine diphospho L rhamnose
EDTA	ethylenediaminetetra acetic acid
e g	for example
EPS	extracellular polymeric substances
Fig	figure
x *g*	centrifugal force
gfp	green fluorescent protein gene
GFP	green fluorescent protein
Gm	gentamicin
Gm^R	gentamicin resistance
h	hour
HPLC/MS	high performance liquid chromatography/mass spectrometry
IPTG	isopropyl β D thiogalactoside
Km^R / kan^R	kanamycin resistance
kb	kilobase pairs
kDa	kilodalton
LB broth	Luria Bertani broth

L	litre
LPS	lipopolysaccahride
M	molar
MCS	multiple cloning site
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mU	milli unit
nm	nanometer
Nm	neomycin
OD	optical density
$3OC_{12}$ HSL	N (3 oxododecanoyl) L homoserine lactone
OHHL	N (3 oxohexanoyl) homoserine lactone
ORF	open reading frame
PCR	polymerase chain reaction
Pers Comm	personal communication
pmol	picomole
qsc	quorum sensing controlled
rpm	revolutions per minute
σ factor	sigma factor
s	second
SDS	sodium dodecyl sulphate
TE	Tris EDTA
Tc	tetracycline
Tc^R	tetracycline resistance
U	units
µg	microgram
µl	microlitre
µM	micromolar
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
X gal	5 bromo 4 chloro 3 indolyl β D galactopyranoside

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

1 1 GENERAL INTRODUCTION

Pseudomonas aeruginosa is a motile aerobic gram negative rod shaped bacterium that is found in a variety of environments *P aeruginosa* is also an opportunistic human pathogen causing serious infection in immunocompromised patients such as those with AIDS as well as patients suffering from cystic fibrosis and severe burns (Cross *et al* 1983 Mendelson *et al* 1994 Richard *et al* 1994 Dunn and Wunderink 1995 Bergen and Shelhamer 1996 Govan and Deretic 1996) The success of *P aeruginosa* to survive and thrive in these diverse environments can be ascribed to its broad metabolic versatility the production of various cell associated and secreted virulence factors that include amongst others exotoxin A elastases (LasA and LasB) alkaline protease exoenzyme S phospholipase C and rhamnolipid (Van Delden and Iglewski 1998) together with properties such as adherence biofilm formation and resistance to antibiotics The latter may be ascribed to the low permeability of the outer membrane efficient multidrug efflux pumps and the production of β lactamases (Hancock and Speert 2000) Expression of many of the above extracellular virulence factors is regulated in a cell density dependant manner also known as quorum sensing

P aeruginosa has at least two quorum sensing systems that interact with each other The first one to be identified was the *lasR/lasI* system (Passador *et al* 1993) which was followed by identification of the *rhlR/rhlI* system (Ochsner *et al* 1994) The LasR protein enhances the transcription of genes that encode extracellular virulence factors including alkaline protease exotoxin A and the LasA and LasB elastases (Swift *et al* 2001) The *rhl* genes are involved in the synthesis of rhamnolipid which is thought to be a virulence factor by virtue of its ciliostatic effect on respiratory cilia (Hingley *et al* 1986) The RhlR protein also regulates the production of chitinase cyanide and pyocyanin (Swift *et al* 2001) The LasR protein seems to stimulate the production of a third quorum sensing molecule that is a quinolone (2 heptyl 3 hydroxy 4 quinolone) also called PQS (Pesci *et al* 1999) PQS may not be involved in quorum sensing directly but may serve as an intermediate messenger between the *las* and *rhl* systems

In this review of the literature aspects relating to *P aeruginosa* biofilm formation quorum sensing systems and rhamnolipid biosynthesis will be addressed More detailed information related to the specific aims of this study have been included under the extended Introductions of the individual Chapters in this dissertation

1 2 BIOFILM FORMATION BY *Pseudomonas aeruginosa*

The phenomenon that marine bacteria grow for the most part on submerged surfaces rather than being free floating (planktonic) was recognised as early as 1933 (Henrici 1933) With the rediscovery that bacteria are found predominantly attached to surfaces in aquatic systems (Geesey *et al* 1977) much attention has been paid to unravelling the molecular mechanisms underlying the formation and regulation of biofilms The formation of well developed biofilms (Fig 1 1) occurs in a sequential process of transport of microorganisms to a surface initial attachment to the surface by bacterial cells formation of microcolonies and formation of well developed biofilms (Molin and Tolker Nielsen 2003)

Prior to surface colonisation a preconditioning film composed of proteins glycoproteins and organic nutrients is believed to form on the attachment surface upon its immersion in liquid (Marshall *et al* 1971) Planktonic bacteria may be transported to the conditioned surface by either a random (e g liquid flow or sedimentation) or in a directed manner (e g chemotaxis and active motility) (Quirymen *et al* 2000) Many studies have shown that surface appendages such as flagella pili and adhesins play a role in bacterial interaction with the surface (Hoyle *et al* 1993 O Toole and Kolter 1998 Costerton *et al* 1995 Vidal *et al* 1998) Whereas the primary function of flagella in biofilm formation is presumed to be transport and initial cell to surface contact pili and pilus associated adhesins are reported to be important for the adherence to and colonisation of the surface In addition to the roles played by flagella and pili initial attachment of the bacteria to the pre conditioned surfaces is also facilitated by electrostatic and hydrophilic interactions as well as Van der Waals forces (Zottola and Sasahara 1994 An *et al* 2000)

Following initial attachment the attached bacterial cells produce extracellular polymeric substances (EPS) that result in a more stable attachment by forming organic bridges between the cells and the substratum (Notermans *et al* 1991 Jones *et al* 1995 Henzer *et al* 2001) Once the bacteria have irreversibly attached to the surface subsequent growth and multiplication of the primary colonising bacteria lead to the formation of microcolonies Under favourable environmental conditions the microcolonies may develop into well developed biofilms which have mushroom like structures interspersed with fluid filled channels (Costerton *et al* 1995) Cells that are loosely attached or that are the furthest from the surface may detach from the biofilm once critical mass and a dynamic equilibrium has been reached These cells together with offspring of other biofilm cells may

colonise previously uncolonised surfaces thereby extending the spatial boundaries of the biofilm (Heydorn *et al* 2002) Environmental conditions such as diffusion of oxygen and antimicrobials in the biofilm (de Beer *et al* 1994 Stewart 2003) carbon source (Klausen *et al* 2003a b) surface or interface properties nutrient availability and hydrodynamics in the biofilm (Purevdorj *et al* 2002) may also aid in detachment A recent study by Webb *et al* (2003) indicated that prophage mediated cell lysis and cell dispersal may also contribute to void formation inside a microcolony

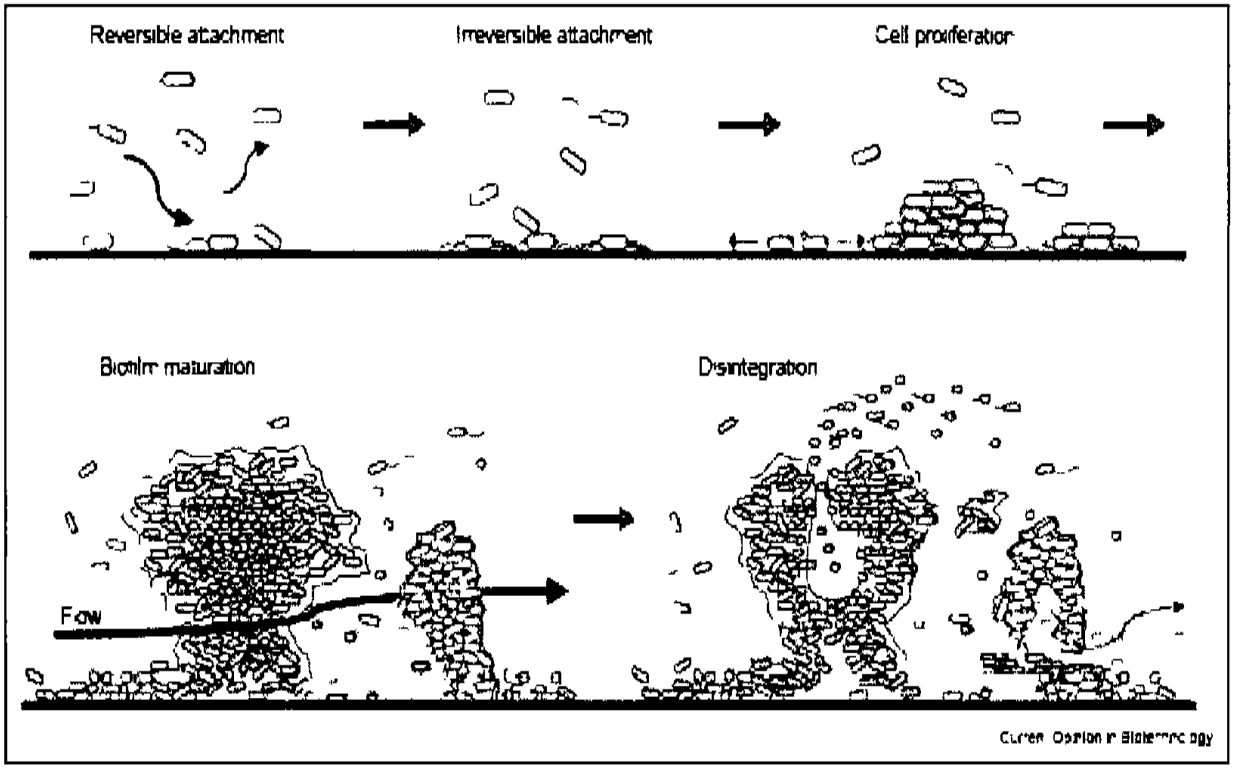


Fig 1 1 Biofilm formation begins with attachment of single cells to a surface This first step which is often reversible may require active swimming motility or be caused by random contacts In a second phase the attachment is fixed by adherence of the cells to the substratum through surface appendages or production of extracellular polymeric substances The third phase represents growth of the attached cells into microcolonies In a hydrodynamic environment the development of the microcolonies depends on cell cell binding interactions The fourth stage is referred to as the mature biofilm At this point the biofilm structure with its distribution of biomass and the presence of water filled voids illustrates the heterogeneity of the biofilm and the rigid properties of the developed structure The stability of the biofilm is secured partly by cell cell interactions and partly by the extracellular polymeric substances matrix surrounding and integrated into the biomass of the biofilm At some point the biofilm may partially dissolve releasing cells that may move away to other locations where a new cycle can begin (Taken from Molin and Tolker Nielsen 2003)

1 3 QUORUM SENSING

Quorum sensing is commonly used to describe the phenomenon whereby the accumulation of a low molecular mass signalling molecule enables individual cells to sense when the minimal population unit or quorum of bacteria has been achieved for a concerted action to be initiated (Hastings and Nealson 1977) This system relies on two major components a small diffusible signalling molecule which accumulates in a population density dependant manner and a transcriptional activator protein which in concert with a signalling molecule activates the expression of relevant genes

1 3 1 The basic quorum sensing system

One of the most intensely investigated quorum sensing systems is the regulation of bioluminescence in *Vibrio fischeri* (Nealson *et al* 1970 Eberhard 1972) *V fischeri* lives both as a symbiont in the light organs of certain marine fish and squid and as a planktonic organism in sea water When at low cell density in sea water *V fischeri* cultures appear dark In contrast when at high cell density within the light organs the population emits light and becomes bioluminescent (Eberhard 1972) In *V fischeri* bioluminescence is dependant on the accumulation of an autoinducer namely N (3 oxohexanoyl) homoserine lactone (OHHL) (Eberhard *et al* 1981) The bioluminescent gene cluster (*lux*) of *V fischeri* consists of eight genes (*luxA E luxG luxI* and *luxR*) (Engebrecht and Silverman 1984) Whereas *luxI* is required for autoinducer synthesis *luxCDABEG* are required for light production and *luxR* encodes a transcriptional activator At low cell densities *luxI* is expressed at basal level and OHHL accumulates in the medium until it reaches a sufficient concentration It is then thought to interact with LuxR forming a complex which then binds to the *lux* promoter region upstream of *luxI* known as the *lux* box and strongly stimulates transcription of the *luxICDABEG* operon This causes an induction of luminescence and generates a positive feedback loop leading to further expression at *luxI* and more OHHL (Fuqua *et al* 1994 Stevens *et al* 1994 Sitnikov *et al* 1995 Stevens and Greenberg 1997)

1 3 2 The *las* quorum sensing system of *P aeruginosa*

In recent years the microorganism on which most quorum sensing related studies have been performed is *P aeruginosa* Two systems *las* and *rhl* have been identified in this bacterium As with the *lux* system of *V fischeri* the *P aeruginosa* regulator inducer complex binds to a consensus sequence (NNCT N_{12} AGNN) upstream of the promoter region in this case known as the *lux* like

box (Whiteley and Greenberg 2001) The first quorum sensing system described in *P aeruginosa* was named the *las* system (Passador *et al* 1993) The *las* cell to cell signalling system is composed of the *lasI* gene which encodes for an autoinducer synthetase responsible for the synthesis of *N* (3 oxododecanoyl) L homoserine lactone ($3OC_{12}$ HSL) and the *lasR* gene which encodes a transcriptional activator The *las* system primarily regulates expression of the LasB elastase (*lasB*) (Gambello and Iglewski 1991 Pearson *et al* 1994) as well as other virulence factors (Fig 1 2) such as LasA protease (*lasA*) alkaline protease (*apr*) and exotoxin A (*toxA*) (Gambello *et al* 1993 Passador *et al* 1993 Pearson *et al* 1997) The *las* system also auto regulates *lasI* leading to higher levels of autoinducer and in turn increases the amount of $3OC_{12}$ HSL that is available to bind to LasR (Pearson *et al* 1997) This results in an increase in expression of the virulence genes once a critical cell density has been reached (Seed *et al* 1995) The *las* system has also been shown to activate the *xcpP* and *xcpR* genes that encode proteins of the *P aeruginosa* secretory pathway (Chapon *et al* 1997)

1 3 3 The *rhl* quorum sensing system of *P aeruginosa*

The second quorum sensing system discovered in *P aeruginosa* was the *rhl* system (Ochsner *et al* 1994) The *rhl* system is composed of the *rhlI* gene which encodes the autoinducer synthetase responsible for the synthesis of *N* butyryl L homoserine lactone (C_4 HSL) (Ochsner and Reiser 1995) The *rhlRI* genes are organised in the *rhlABRI* regulon and the *rhlAB* genes are regulated by a single promoter (Ochsner and Reiser 1995) The *rhl* quorum sensing system primarily regulates the expression of the *rhlAB* operon encoding for rhamnosyltransferase 1 which is responsible for the synthesis of mono rhamnolipid (Ochsner and Reiser 1995) Other virulence factors that have been shown to be regulated by the *rhl* quorum sensing system (Fig 1 2) include pyocyanin cyanide lipase C LasB *(lasB*) and LasA (*lasA*) elastases and alkaline protease (*aprA*) (Brint and Ohman 1995 Pearson *et al* 1997 Reimmann *et al* 1997) Recently the mechanism by which RhlR regulates and binds to the *rhlAB* promoter region has been elucidated RhlR was found to bind the *rhlAB lux* like box in the presence and absence of the autoinducer When it binds to the *lux* like box in the absence of the autoinducer the expression of *rhlAB* is repressed but when the RhlR(C_4 HSL) complex binds to the *lux* like box the transcription of the *rhlAB* genes is activated by direct interaction with the RNA polymerase (Medina *et al* 2003a)

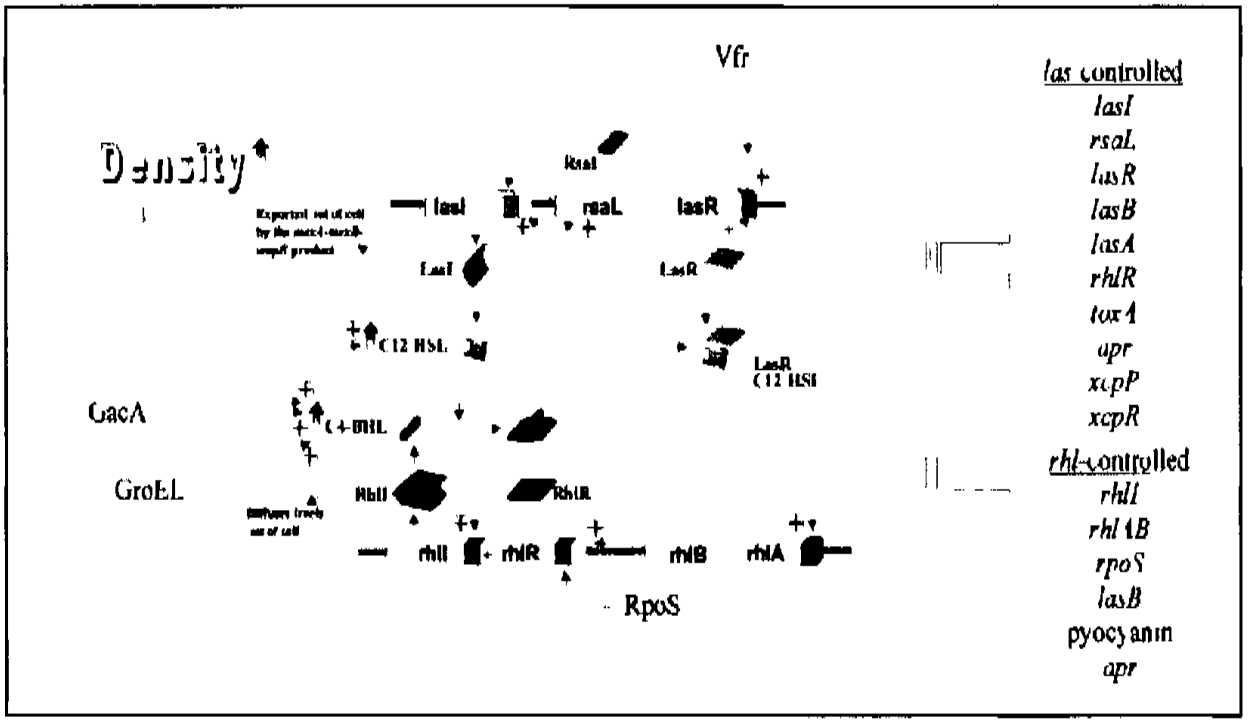


Fig 1 2 Model of the *las/rhl* quorum sensing system in *Pseudomonas aeruginosa* Virulence factors controlled by the *las* and *rhl* quorum sensing systems are shown to the right of the figure (Taken from Shirtliff *et al* 2002)

1 3 4 The quorum sensing hierarchy in *P aeruginosa*

Various reports have shown that the *las* and the *rhl* quorum sensing systems of *P aeruginosa* interact Both systems are highly specific in that their respective autoinducers are unable to activate the transcriptional activator protein of the other system e g $3OC_{12}$ HSL cannot activate RhlR and C_4 HSL cannot activate LasR (Latifi *et al* 1995 Pearson *et al* 1997) However neither system is completely independent of the other The LasR($3OC_1$ HSL) complex activates the expression of *rhlR* thus placing the *las* system in quorum sensing hierarchy above the *rhl* system (Latifi *et al* 1996 Pesci *et al* 1997) Moreover $3OC_{12}$ HSL can bind to RhlR thus blocking the binding of C_4 HSL to its transcriptional activator RhlR (Pesci *et al* 1997) The *las* quorum sensing system therefore controls the *rhl* system at both the transcriptional and posttranslational level

1 3 5 Relevance of quorum sensing

Analysis of the *P aeruginosa* transcriptome (Schuster *et al* 2003 Wagner *et al* 2003) has led to the identification of 315 quorum induced and 38 quorum repressed genes representing approximately 6% of the *P aeruginosa* genome (Schuster *et al* 2003) Furthermore proteome analysis of the extracellular proteins from quorum sensing mutants of *P aeruginosa* indicated a

total of 13 quorum sensing gene products of which seven have not previously been associated with quorum sensing (Nouwens *et al* 2003) Several proteins such as FlgK FliC PhoA AotJ DsbA and GlpQ have been shown to be down regulated in the quorum sensing mutant The *las* and *rhl* quorum sensing systems are also regulated by various global regulators or efflux systems e g *nfxC* (Kohler *et al* 2001) Vfr (Beatson *et al* 2002) GacA (Parkins *et al* 2001) RpoS (Latifi *et al* 1996) AlgQ (Ledgham *et al* 2003a) DksA (Jude *et al* 2003) QscR (Ledgham *et al* 2003b) MvaT (Diggle *et al* 2002) and RpoN (Thompson *et al* 2003 Heurlier *et al* 2003) The various genes under the control of the *las* and *rhl* quorum sensing systems and the multiple regulatory levels highlight the importance of these systems for *P aeruginosa* The role of RpoN and RpoS in the regulation of rhamnolipid synthesis is discussed in more detail in Chapter 4

An early report by Davies *et al* (1998) suggested that the formation of a well developed biofilm by *P aeruginosa* was dependent on the synthesis of $3OC_{12}$ HSL The *P aeruginosa lasI* mutant lacked the three dimensional architecture of the wild type biofilm However a later study (Heydorn *et al* 2002) indicated that there where no difference in biofilm structure or development between the wild type *P aeruginosa* and the *lasI* mutant This discrepancy can possibly be attributed to the recent availability of statistical software developed by the authors (Heydorn *et al* 2000) to compare and analyse various images obtained from the confocal laser scanning microscope (CLSM) to determine if a specific mutation or growth condition has a significant effect on the development of the biofilm in question Furthermore the authors also referred to other studies supporting their findings and concluded that the *lasI lasR* quorum sensing system was not required for the development and formation of mature *P aeruginosa* biofilms

Quorum sensing and quorum sensing controlled genes also appear to be important for the pathogenic potential of *P aeruginosa* Bacterial strains isolated from urinary catheters (Stickler *et al* 1998) and contact lenses (Zhu *et al* 2001) have been reported to produce homoserine lactones when they are grown on agar plates *P aeruginosa* in sputum samples from cystic fibrosis patients is clustered in biofilm like groupings (Singh *et al* 2000) The ratios of C_4 HSL to total homoserine lactones were lower in broth cultures than in artificial biofilms Notably the same ratio seen in artificial biofilms was also seen in the sputum samples from cystic fibrosis patients thus providing strong evidence that biofilms are relevant in cystic fibrosis infections Furthermore mutant strains of *P aeruginosa* of which the *lasR* had been inactivated have been reported to be much less efficient in causing bacteremia and failed to kill neonatal mice that had been inoculated with the

bacteria In contrast the wild type strain was three times more likely to cause bacteremia (Tang *et al* 1996) Similar results have been obtained in a rat model of pneumonia using *P aeruginosa* deficient in both the *lasI* and *rhlI* genes (Wu *et al* 2001) Bacteria lacking quorum sensing synthetases produced a milder pneumonia and were able to survive in smaller numbers than their isogenic parental strains

1 4 BIOSURFACTANTS AND RHAMNOLIPID

1 4 1 Definitions and classifications

A biosurfactant is defined as a surface active molecule produced by living cells in the majority of cases microorganisms (Fiechter 1992 Desai and Banat 1997) The main physiological role of biosurfactants is to permit microorganisms to grow on water immiscible substrates by reducing the surface tension at the phase boundary thus making the substrate more readily available for uptake and metabolism Direct uptake of hydrocarbons dissolved in the aqueous phase direct contact of cells with large hydrocarbon droplets as well as interaction with solubilised droplets (emulsion) have been described (Fiechter 1992) In addition to emulsification of the carbon source biosurfactants are also involved in the adhesion of microbial cells to the hydrocarbon The cellular adsorption of the hydrocarbon degrading microorganisms to water immiscible substrates and the excretion of surface active compounds together allow growth on such carbon sources (Kappeli and Fiechter 1976) Apart from their physiological importance biosurfactants has also found numerous industrial and environmental applications Surfactants are often used for their detergency emulsifying foaming and dispersing traits (Desai and Banat 1997) The worldwide surfactant market exceeds $9 4 billion per year (Desai and Banat 1997) Although the surfactants marketed are almost exclusively synthetic the interest in microbial surfactants has been increasing over the last decade This has been due to their diversity environmental friendliness and the possibility of producing them more economically through fermentation In contrast to synthetic surfactants microbial surfactants are less toxic display higher biodegradability are environmentally compatible (i e non toxic and biodegradable) and display high selectivity and specific activity at extreme temperatures pH and salinity (Desai and Banat 1997)

The surfactant character of molecules is due to their mixed hydrophilic/hydrophobic nature They are able to form micelles and reversed micelles or to aggregate to form rod shaped micelles bilayers and vesicles (Desai and Banat 1997) They accumulate at interfaces and mediate between

phases of different polarity such as oil/water air/water or water/solid acting as wetting agents on solid surfaces This dynamic process is based on the ability of the surfactant to reduce the surface tension by governing the arrangement of liquid molecules thus influencing the formation of hydrogen bonds and hydrophobic hydrophilic interactions Minimum surface tension and the critical micelle concentration (cmc) are parameters used to measure the efficiency of the surfactant

Biosurfactants may be classified into three main groups based on the detail of their chemical structure within the basic framework whereas the hydrophobic moiety consists of the hydrocarbon chain of a fatty acid the hydrophilic moiety may consist of the ester or alcohol function of neutral lipids or the carboxylate group of fatty acids or amino acids or the phosphate containing portions of phospholipids or the carbohydrate moiety of glycolipids (Fiechter 1992)

The glycolipid surfactants

These include the sophorose rhamnose trehalose sucrose and fructose lipids from a range of species such as *Torulopsis* (Ito and Inoue 1982) *Pseudomonas* (Syldatk *et al* 1985) or *Arthrobacter* (Shabtai and Gutnick 1986) Among the best investigated biosurfactants of this group are the rhamnolipids from *P aeruginosa* Under specific environmental conditions *P aeruginosa* produces and secretes rhamnose containing glycolipid biosurfactants called rhamnolipids Rhamnolipid can be described as one or two molecules of rhamnose linked to one or two molecules of β hydroxy decanoic acid via a glycosidic linkage (Lang and Wullbrandt 1999) The most common structural variants of rhamnolipid are indicated in Fig 1 3

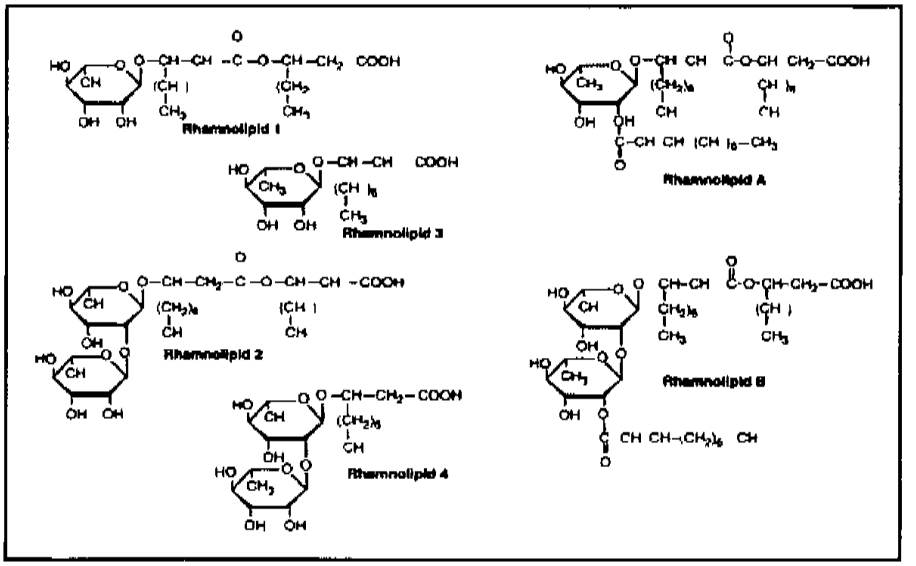


Fig 1 3 Structural variants of rhamnolipid (Taken from Lang and Wullbrandt 1999)

The amino acid containing lipid biosurfactants

This group includes surfactin which is produced by *Bacillus subtilis* and is one of the most powerful biosurfactants (Arima *et al* 1968 Schaller *et al* 2004) Surfactin is composed of a seven amino acid ring structure coupled to one molecule of 3 hydroxy 13 methyl tetradecanoic acid (Peypoux *et al* 1999) Frequently biosurfactants in this group have been noted for their antibiotic activity with the surfactant properties having been of only minor interest

Biosurfactants containing polysaccharide lipid complexes

This group of biosurfactants contains the polysaccharide lipid complexes of *Candida tropicalis* and *Acinetobacter calcoaceticus* RAG 1 The latter organism is the source of Emulsan an extracellular lipo heteropolysaccharide polyanionic bioemulsifier (Shabtai and Gutnick 1986) The majority of the polysaccharides that are located predominantly in the outer membrane of gram negative bacteria exert surfactant activities (Fiechter 1992) The cell walls of yeast such as the alkane assimilating *Candida tropicalis* have been shown to contain a polysaccharide lipid complex related to alkane transfer through the cellular membrane (Kapelli and Fiechter 1977) The biosynthesis of the polysaccharide lipid complex is induced by growing the yeast on hydrocarbons

Protein like substances

A number of protein like substances with biosurfactant activity have been identified A 70 kDa protein called serraphobin isolated from both the cell surface and culture supernatants of *Serratia marcescens* is able to bind to hexadecane droplets (Bar Ness and Rosenberg 1989) The yeast *Candida lipolytica* produces liposan a 27 6 kDa protein like substance composed of 83% carbohydrate that is capable of stabilising water/oil emulsions (Cirigliano and Carmon 1985) Similar protein carbohydrate complexes which act as emulsifiers have been found in *P fluorescens* and *P aeruginosa* (Fiechter 1992 Patel and Desai 1997)

1 4 2 Rhamnolipid synthesis pathway

As previously mentioned rhamnolipid is one of the many virulence factors produced by *P aeruginosa* and its expression is coordinately regulated at the transcriptional level by two quorum sensing systems The production of rhamnolipids by *P aeruginosa* was first reported by Jarvis *et al* in 1949 whilst the fact that they are secreted by the bacteria in the stationary phase of growth was described almost 50 years ago (Hauser and Karnovsky 1958)

In liquid culture *P aeruginosa* primarily produces two forms of rhamnolipid (Fig 1 4) namely rhamnosyl β hydroxydecanoyl β hydroxydecanoate (mono rhamnolipid) and rhamnosyl rhamnosyl β hydroxydecanoyl β hydroxydecanoate (di rhamnolipid) (Rendell *et al* 1990) The biosynthesis of these two forms of rhamnolipid proceeds by two sequential rhamnosyl transfer reactions each catalysed by a specific rhamnosyltransferase (Rt 1 and Rt 2 respectively) with deoxy thymidine diphospho L rhamnose (dTDP L rhamnose) acting as the rhamnosyl donor in both reactions and β hydroxydecanoyl β hydroxydecanoate or mono rhamnolipid acting as the respective recipients (Burger *et al* 1962)

The rhamnosyltransferase 1 enzyme is composed of two polypeptides that are encoded by the *rhlA* and *rhlB* genes which are organised as an operon (Ochsner *et al* 1994) The RhlA protein (32 5 kDa) seems to play an important role in the synthesis of rhamnolipids but its exact function remains to be determined It has been suggested that the RhlA protein is necessary for the stabilisation of RhlB in the cytoplasmic membrane More recent evidence however indicates that RhlA is probably involved in the synthesis of 3 hydroxyacyl ACP precursors that are common intermediates in the biosynthesis of polyhydroxyalkanoic acids e g β hydroxydecanoyl β hydroxydecanoate (Rehm *et al* 1998) One report suggested that RhlA catalyses the reaction of β hydroxydecanoyl ACP to β hydroxydecanoyl S CoA (Deziel *et al* 2003) In contrast RhlB (47 kDa) is hypothesised to be the catalytic subunit of the rhamnosyltransferase and contains two putative membrane spanning domains that are thought to anchor RhlB in the inner membrane (Ochsner *et al* 1994)

The *rhlC* gene encoding the rhamnosyltransferase 2 is homologous to rhamnosyltransferases involved in lipopolysaccharide (LPS) biosynthesis (Rahim *et al* 2001) RhlC is involved in the synthesis of di rhamnolipid (Fig 1 4) and similar to *rhlAB* it is also under control of the *rhl* quorum sensing mechanism (Rahim *et al* 2001) Rahim and co workers have also proposed a putative rhamnolipid biosynthesis pathway It has been proposed that the assembly of the precursors dTDP L rhamnose and β hydroxydecanoyl β hydroxydecanoate into mono rhamnolipid is catalysed by the RhlAB complex in close proximity to the cytoplasmic membrane whereafter some of the mono rhamnolipid is directly secreted from the cell while RhlC transforms another portion into di rhamnolipid by using the dTDP L rhamnose moiety as a donor The di rhamnolipid is then secreted from the cell by an as yet unknown mechanism (Rahim *et al* 2001)

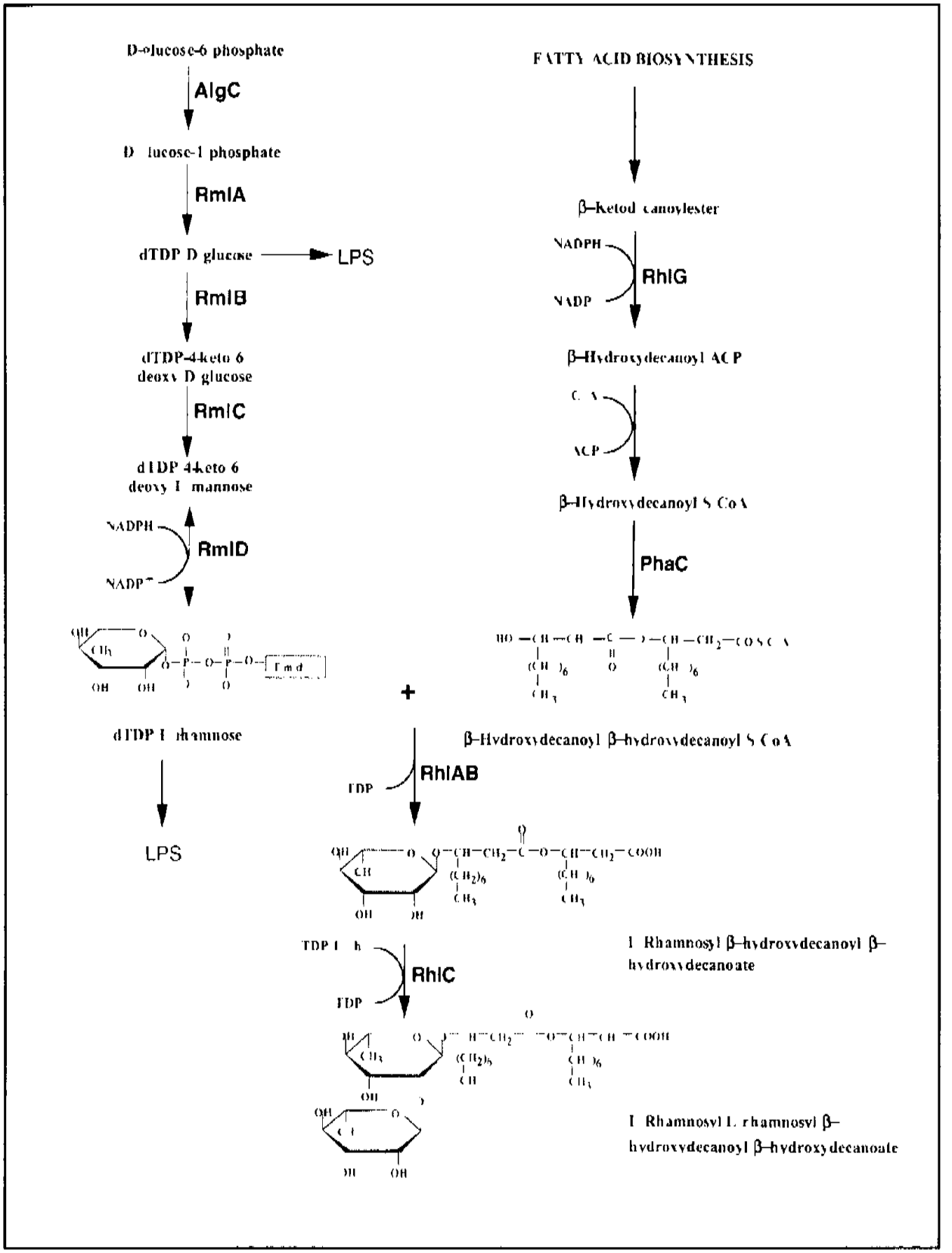


Fig 1.4 *Pseudomonas aeruginosa* rhamnolipid biosynthesis pathway (Taken from Maier and Soberon-Chavez 2000)

The first enzyme reported to be involved in the synthesis of the fatty acid moiety of rhamnolipid was RhlG and it was also reported that the pathway for the synthesis of this moiety separates from the *P aeruginosa* general fatty acid biosynthetic pathway at the level of the ketoacyl reduction (Campos Garcia *et al* 1998) The RhlG enzyme is thus responsible for draining the fatty acid precursors of rhamnolipid from the general biosynthetic pathway and shows sequence homology with NADPH dependent ketoacyl reductases

The donor of the rhamnosyl moiety for the synthesis of both mono and di rhamnolipid is dTDP L rhamnose which is also the L rhamnose donor for the synthesis of all cell polymers containing this sugar In *P aeruginosa* L rhamnose is a common component of the LPS O antigen (Rochetta *et al* 1999) The genes (*rmlABCD*) encoding the enzymes involved in the synthesis of TDP L rhamnose from glucose 1 phosphate (Fig 1 4) are typically arranged as an operon and have been described in several bacteria (Koplin *et al* 1993 Zhang *et al* 1994 Macpherson *et al* 1994)

The *algC* gene encodes a phosphomannomutase catalysing the second step in the alginate biosynthetic pathway i e the conversion of mannose 6 phosphate to mannose 1 phosphate This enzyme has also been shown to have a phosphoglucomutase activity and to be involved in LPS synthesis (Coyne *et al* 1994) presumably participating in the biosynthesis of glucose and rhamnose needed for the formation of core LPS Olvera *et al* (1999) reported that AlgC plays a central role in the production of rhamnolipids since the enzyme through its phosphoglucomutase activity is responsible for the production of glucose 1 phosphate the precursor of dTDP glucose and ultimately of dTDP L rhamnose (Fig 1 4)

Rhamnolipids can be commercially produced at levels of up to 100 g/L (Lang and Wullbrandt 1999) Since *P aeruginosa* is an opportunistic human pathogen and therefore does not have GRAS status attempts have been made to produce rhamnolipid in heterologous expression hosts However attempts to produce rhamnolipid in recombinant nonpathogenic pseudomonads or in *E coli* have thus far been unsuccessful and very low amounts of the surfactants are produced (Ochsner *et al* 1994 1995) The main reason for this is that not all of the genes involved in the synthesis pathway of rhamnolipid is known The substrates of the rhamnosyltransferase 1 and 2 enzymes are compounds produced by central metabolic pathways (Fig 1 4) Thus in order to produce high levels of rhamnolipid in heterologous hosts a metabolic engineering strategy would be needed that would enable overproduction of TDP L rhamnose and β hydroxy fatty acids Additionally the

complexity of the *P aeruginosa* regulatory network involved in rhamnolipid production furthermore complicates high level expression of rhamnolipid in heterologous hosts

1 4 3 Applications of rhamnolipids

The increasing interest in the potential applications of microbial surface active compounds is based on their broad range of functional properties that include emulsification phase separation wetting foaming solubilisation corrosion inhibition and viscosity reduction (e g of heavy crude oils) Rhamnolipids have been used in various different applications e g to clean up marine oil pollution (Harvey *et al* 1990) soil remediation (Finnerty 1994) decontamination of heavy metals such as Pb^{2+} Zn and Cd^{2} from the environment (Tan *et al* 1994 Herman *et al* 1995 Betts 1997 Maslin and Maier 2000) and it has been used successfully as a soil washing or flushing agent (Scheibenbogen *et al* 1994 Bai *et al* 1997) Rhamnolipid has also shown promise as a biological control agent against several genera of zoosporic plant pathogens such as *Pythium aphanidermatum Phytophthora capsici* and *Plasmopara lactucae radicis* (Stanghellini and Miller 1997) Purified mono and di rhamnolipid in concentrations ranging from 5 to 30 g/L caused cessation of motility and lysis of the entire zoospore population

Amongst the many potential industrial and environmental applications of rhamnolipid (Fiechter 1992 Miller 1995 Desai and Banat 1997 Schaller *et al* 2004) the most promising appears to be related to its ability to degrade various organic compounds Addition of rhamnolipid to pure cultures has been shown to enhance the biodegradation of several different hydrocarbons both aliphatic and aromatic (Oberbremer *et al* 1990 Zhang and Miller 1992 1995 Miller 1995 Providenti *et al* 1995) Rhamnolipids are suggested to have two mechanisms by which they enhance degradation The first is to increase hydrocarbon solubility thereby increasing the bioavailability to degrading cells (Zhang and Miller 1992 Shreve *et al* 1995) The second is to interact with the degrading cell causing the cell surface to become more hydrophobic and associate more easily with hydrophobic substrates (Shreve *et al* 1995 Zhang and Miller 1994) Further investigation of the mechanism of interaction of rhamnolipid with the *Pseudomonas* cell surface has revealed that rhamnolipids cause a loss of LPS an important hydrophilic component of the cell surface (Al Tahhan *et al* 2000) Loss of LPS results in an increase in the relative hydrophobicity of the cell The second mechanism mentioned above may however be more important for *in situ* bioremediation of hydrocarbons (Herman *et al* 1997) This is because while high levels of rhamnolipid are required to substantially increase hydrocarbon solubility only low levels of

rhamnolipid are required to alter the cell surface The advantages of this is two fold First because rhamnolipids are biodegradable they can serve as a preferred carbon source in a mixed population In this case addition of high amounts of rhamnolipid would suppress degradation of hydrocarbons until a substantial portion of the rhamnolipid has been degraded Second lower levels of rhamnolipid are less apt to cause mobilisation of hydrocarbons which could result in undesirable spreading of a contaminant plume (Herman *et al* 1997)

Microbes offer a largely unexplored source of rhamnolipids with many potential biotechnological applications but it may be difficult to realise their full application (Maier and Soberon Chavez 2000 March and Bentley 2004) Firstly product efficacy must be demonstrated after which the new microbial product must be produced at an economical scale and yield Finally they must then be tested to meet regulatory guidelines Although rhamnolipid is commercially produced in high yield and in large scale fermenters making it economically competitive with synthetic surfactants much effort is still needed for process optimisation at engineering and biological levels Characterization of rhamnolipids their biosynthetic pathways and their regulatory networks is especially needed

1 5 AIMS OF THIS INVESTIGATION

In *P aeruginosa* the production of several virulence associated factors including rhamnolipid is coordinately regulated by quorum sensing (Van Delden and Iglewski 1998) The quorum sensing response depends on the production of two autoinducers *N* butyryl L homoserine lactone (C_4 HSL) and *N* (3 oxododecanoyl) L homoserine lactone ($3OC_{12}$ HSL) which coupled with the proteins RhlR and LasR respectively activate gene expression The transcriptional activator RhlR coupled with C_4 HSL (Ochsner and Reiser 1995) promotes the expression of amongst other the *rhlAB* operon that encodes the enzyme rhamnosyltransferase 1 (Ochsner *et al* 1994 Pearson *et al* 1997 Pesci *et al* 1997) and the *rhlC* gene which encodes rhamnosyltransferase 2 (Rahim *et al* 2001) The *rhlAB* and *rhlC* encoded rhamnosyltransferases are responsible for mono and di rhamnolipid biosynthesis respectively In addition to the quorum sensing systems global regulators such as Vfr (Beatson *et al* 2002) GacA (Parkins *et al* 2001) and RpoS (Suh *et al* 1999) have also been shown to regulate amongst other rhamnolipid production as well as surface attachment and biofilm development Furthermore it has been reported that alginate lipase type IV fimbrae and rhamnolipid are all regulated by RpoN (Studholme *et al* 2000 Heurlier *et al* 2003 Thompson *et*

al 2003) In addition to contributing to the pathogenic potential of *P aeruginosa* it has been reported that *P aeruginosa* utilises rhamnolipid to actively maintain the void spaces surrounding microcolonies (Davey *et al* 2003) These void spaces or channels not only allow fluids to flow throughout the biofilm resulting in the distribution of nutrients and oxygen but they may also provide a means of removing metabolic end products (Molin and Tolker Nielsen 2003) Since our research group is interested in the mechanisms underlying *P aeruginosa* biofilm development and based on the role of rhamnolipid in maintaining the biofilm architecture the aims of this study were as follow

Primary aims of this study

(a) To develop and optimise a biochemical assay for rhamnolipid extraction and quantitation

(b) To generate a *rhlAB* deficient mutant strain of *P aeruginosa* PAO1 by directed insertional mutagenesis using an appropriate allelic exchange vector

(c) To investigate the transcriptional activity of the *P aeruginosa rhlAB* promoter in different mutant strains and under different growth conditions using reporter gene technology

The research strategies for obtaining the primary objectives involved the following

Optimisation of the anthrone assay whereby rhamnolipid produced by *P aeruginosa* PAO1 could be quantified using small sample volumes of the culture supernatant (Chapter 2)

Construction of different allelic exchange vectors and evaluation of different approaches whereby the *rhlAB* genes of *P aeruginosa* PAO1 can be specifically inactivated (Chapter 3)

Preparation of *gfp* transcriptional fusions and analysis of the transcriptional activity of *rhlAB* in RpoS and RpoN deficient mutants strains of *P aeruginosa* PAO1 under different growth conditions as well as in developing and well developed biofilms respectively (Chapter 4)

CHAPTER 2

ESTABLISHMENT OF A METHOD TO DETERMINE THE CONCENTRATION OF RHAMNOLIPID IN SMALL SAMPLE VOLUMES FROM A *Pseudomonas aeruginosa* PAO1 CULTURE SUPERNATANT

2 1 INTRODUCTION

Pseudomonas aeruginosa produces and secretes rhamnose containing glycolipid biosurfactants called rhamnolipids of which the molecular structure comprise a hydrophilic portion composed of monosaccharides and a hydrophobic portion composed of β hydroxy decanoic acids (Jarvis and Johnson 1949) Several structural variants of rhamnolipids exist and these contain hydroxy fatty acids of different chain lengths and either one or two rhamnose sugars (Lang and Wullbrandt 1999) Due to their commercial importance a great deal of effort has been put into developing methods whereby biosurfactants and more specifically rhamnolipid can be characterised and their concentration quantified Each of these methods has advantages and disadvantages as will be highlighted in the following sections

A popular method whereby biosurfactant concentration is measured relies on determining the critical micelle concentration (cmc) (Cooper *et al* 1981) In essence this value is a multiplication factor that indicates how many times the concentration of a biosurfactant is in excess to the critical micelle concentration This method is time consuming yields approximate values and is subject to interference by impurities in the sample A widely used method to determine surface or interfacial tension of solutions containing biosurfactants is the du Nouy ring method Here the force required to pull a platinum wire ring through the liquid air or liquid liquid interface is measured These values are then compared to those obtained for standard surfactants such as SDS thus yielding an approximate concentration relative to the standard Although it is accurate easy to use and fairly rapid it does however rely on the use of specialised equipment (Harkins and Alexander 1959) Recently more sophisticated methods have been reported For example high performance liquid chromatography / mass spectrometry (HPLC/MS) can provide accurate information not only on the amounts of the rhamnolipid but also on the presence of various homologs being produced (Deziel *et al* 2000) Using this approach Haba *et al* (2003) reported the identification of up to 11 rhamnolipid homologs in a single batch culture of *Pseudomonas aeruginosa* An alternative approach relies on the use of infra red spectroscopy (Gartshore *et al* 2000) which detects the strong peak formed by the carbonyl bond of most biosurfactants in the infra red spectrum of the compound This method allows for rapid and accurate measurements directly on the growth medium with minimal sample preparation and the presence of hydrocarbons does not interfere with the measurements Both of these approaches however rely on the availability of specialised equipment and requires some expertise in the use of the equipment

In addition to the above methods simpler methods have also been developed that can either be used for qualitative or quantitative rhamnolipid assays Amongst these is the modified drop collapse technique (Bodour and Miller Maier 1998) The assay is performed in a microtitre plate by pipetting a droplet of the rhamnolipid sample onto oil and measuring the diameter of the droplet after 1 min using a stereo microscope and a micrometer Using a biosurfactant of known concentration a standard curve is prepared from which the sample concentration can be determined based on the diameter of the droplet The presence of hydrocarbons in the growth medium may however influence the diameter of the droplet thus leading to inaccurate rhamnolipid concentration determinations Several authors have reported colorimetric assays whereby the concentration of rhamnolipid in different culture media can be determined These methods are based on the extraction of the apolar rhamnolipid from the culture supernatant with an organic solvent after which the rhamnose sugar moiety is released by acid hydrolysis and then detected spectrophotometrically after reaction with orcinol anthrone or thioglycolic acid (Zhang and Miller 1992 Sim *et al* 1997 Deziel *et al* 1996) Although these methods do not require specialised equipment they are however time consuming and do not distinguish between different rhamnolipids present in a mixed sample

Since our research group is interested in the molecular mechanisms underlying biofilm development and maintenance and based on recent reports indicating that rhamnolipid plays an important role in the maintenance of the biofilm architecture of *P aeruginosa* (Davey *et al* 2003) the aim of this part of the study was to develop an improved methodology for the quantitation of rhamnolipid in small sample volumes (1 ml) Since the colorimetric assays do not rely on the use of specialised equipment these assays were used as a platform from which to develop optimise and refine the rhamnolipid assay described in this part of the investigation

2 2 MATERIALS AND METHODS

2 2 1 Bacterial strain

Pseudomonas aeruginosa PAO1 (Holloway *et al* 1979) was used throughout the course of this study The strain was maintained at 70 C as a glycerol culture (Sambrook *et al* 1989)

2 2 2 Culture conditions for rhamnolipid production

The *P aeruginosa* PAO1 glycerol culture was streaked on PTYG agar containing 5 g peptone 5 g tryptone 10 g yeast extract 0 6 g $MgSO_4$ $7H_2O$ 0 07 g $CaCl_2$ $2H_2O$ and 15 g agar per litre After incubation overnight at 37 C a pre culture was prepared by inoculating a loopfull of bacterial growth into Kay s mineral medium which comprised of 100 ml of Solution A (0 3 g $(NH_4)H_2PO_4$ 0 2 g K_2HPO_4 0 2 g glucose per 100 ml dH_2O) 1 ml of Solution B (0 045 g $FeSO_4$ in 100 ml dH_2O) and 1 ml of Solution C (10 g $MgSO_4$ $7H_2O$ in 100 ml dH_2O) (Pers comm Neilson 1999) The cultures were grown under agitation (200 rpm) at 37 C Following incubation overnight phosphate limited PPGAS medium (0 02 M NH_4Cl 0 02 M KCl 0 12 M Tris HCl 0 5% [w/v] glucose 1% [w/v] proteose peptone 0 0016 M $MgSO_4$ pH 7 2) was inoculated with 1 ml of the pre culture per 100 ml of medium (Zhang and Miller 1992) and incubated as above for up to 5 days Aliquots of the culture were aseptically removed at various time intervals and assayed for rhamnolipid

2 2 3 Rhamnolipid extraction

Rhamnolipid was extracted from small sample volumes using the following optimised method that was developed during the course of this study The cells from 1 ml of the *P aeruginosa* PAO1 culture were pelleted by centrifugation for 5 min at 13 000 x *g* and the supernatant was transferred to a 1 5 ml microfuge tube Rhamnolipid was precipitated by acidifying the supernatant to pH 2 through the addition of 100 µl of concentrated HCl followed by brief vortexing and incubation at 4 C for up to 5 days The solution was then transferred to a 5 ml glass test tube and 2 ml of a chloroform methanol (10 1) mixture was added vortexed for 30 s and incubated at room temperature for 45 min The clear organic (chloroform) phase was carefully recovered and then transferred to a new 5 ml glass test tube The extraction was then repeated once more on the aqueous phase as described above The test tubes containing the chloroform phases were incubated in a water bath at 80 C until the chloroform had evaporated and a thick honey like solution remained at the bottom of the test tubes The rhamnolipid (and other impurities) were then dissolved by addition of 1 ml of 0 05 M sodium bicarbonate (pH 8 6) vortexed briefly and incubated at room temperature for at least 30 min The rhamnolipid was quantified using the anthrone assay as described below

2 2 4 Rhamnolipid quantification

A 10 mM anthrone sulphate (Sigma Chemical Company) solution was prepared in 98% H_2SO_4 and left at room temperature for 1 h to allow complete dissolution The rhamnolipid containing extracts

(Section 2 2 3) were overlayed with twice the volume of anthrone solution by slowly adding the reagent to the samples in glass test tubes on ice The test tubes were vortexed and then heated in a boiling water bath for 15 min (Sim *et al* 1997) After allowing the samples to cool to room temperature a 200 µl aliquot was loaded into a flat bottom microtitre plate (Greiner) and the absorbance measured within 1 h at 620 nm using a Thermo Labsystems Multiskan Ascent microtitre plate reader Distilled water was used to zero the absorbency readings The rhamnolipid concentration (mg/ml) was determined from a standard curve prepared for α L rhamnose (Sigma Chemical Company)

2 2 5 Batch assays

The optical density and culturable count were determined every 2 h for 12 h and then after 24 48 72 96 and 144 h The OD_{605} of 1 ml of culture was determined in a 1 ml cuvette with a path length of 1 cm using a SPECTRONIC® 20 GENESYS spectrophotometer The culturable count was determined by plating 100 µl aliquots of serial ten fold dilutions prepared in dH_2O in triplicate on LB agar plates (1% [w/v] tryptone 0 5% [w/v] yeast extract 1% [w/v] NaCl 1 5% [w/v] agar pH 7 4) (Sambrook *et al* 1989) Following incubation overnight at 37 C the colonies were counted on plates containing between 30 to 300 colonies and then averaged

2 2 6 Detection of glucose in growth medium

The concentration of glucose in PPGAS medium during growth of *P aeruginosa* was determined every 2 h for 12 h and then after 24 48 and 72 h using a commercial kit (GOD PAP Boehringer Mannheim) Briefly the bacterial cells from samples (200 µl) of the culture medium were pelleted by centrifugation at 13 000 x *g* for 5 min and the recovered culture supernatant was mixed with 2 ml of the glucose assay reagent mixture prepared by mixing the contents of vial 1 (containing horseradish peroxidase glucose oxidase and 4 aminophenozone in phosphate buffer) with that of vial 2 (phenol) Following incubation at 25 C for 35 – 60 min away from direct sunlight the absorbance at 510 nm was determined using a SPECTRONIC® 20 GENESYS spectrophotometer Distilled water (200 µl) mixed with the reagent mixture was used to zero the absorbency readings Glucose (Preemat® D Glucose Boehringer Mannheim) assayed as above was used as standard The concentration of glucose (in mg/100 ml) was calculated using the formula $c = 100 \times A_{mpl}/A_{std}$ All assays were performed in triplicate

2 3 RESULTS AND DISCUSSION

During the development of the method for the quantitation of rhamnolipid produced by *P aeruginosa* as described under Materials and Methods (Section 2 2 3) several parameters had to be optimised In this study different culture media and carbon sources were investigated for optimal rhamnolipid production Since previously published papers reported on the extraction of rhamnolipid from large sample volumes (litres) the methodology had to be adapted to the small sample volumes (1 ml) used in this investigation Consequently the use of different solvents and conditions that would allow for maximal rhamnolipid precipitation were also investigated Having optimised the assay it was used to monitor rhamnolipid production by *P aeruginosa* grown in batch The following sections will provide more detailed information regarding the approaches used in developing the assay as well as the results that were obtained

2 3 1 Culture media and carbon sources

Initially *P aeruginosa* was cultured as described by Neilson (1999) which involved pre culturing overnight in Kay s medium prior to inoculation into a mineral salts medium with glucose as carbon source However the mineral salts precipitated from the medium during sterilisation of the medium by autoclaving Therefore *P aeruginosa* was pre cultured in Kay s medium and then rather inoculated into PPGAS medium (Pers comm Soberon Chavez 2000) The bacterial cells are switched from a phosphate rich (Kay s) to phosphate poor medium (mineral salts PPGAS) (Mulligan *et al* 1989) and the available nitrogen is also limited thus resulting in up regulation of rhamnolipid production (Mulligan and Gibbs 1989)

The usefulness of canola oil (Sim *et al* 1997) as a carbon source in mineral salts medium was also investigated However some of the oil in the medium co extracted with the chloroform methanol mixture and cross reacted with the anthrone reagent thus making it impossible to obtain reproducible OD_{620} readings Furthermore investigation of a 48 h old culture by light microscopy indicated that bacterial cells were localised to the oil water interface This would result in co extraction of cellular material including cellular sugars Thus as a consequence of contamination of the rhamnolipid extract with cellular sugars the concentration determined might not be representative of the actual rhamnolipid concentration in the culture medium

2 3 2 Time required for maximal rhamnolipid precipitation

According to Neilson (1999) incubation of the acidified culture supernatant at 4 C for 5 days results in a maximal yield of rhamnolipid Since small sample volumes were used in this study it was investigated whether the time required for rhamnolipid precipitation could be shortened Thus the samples were incubated at 4 C for 0 to 120 h and the yield of rhamnolipid was quantified at various different times An increase in the yield of rhamnolipid over time was recorded and near maximum yield was obtained after 48 h of incubation However as the yield of rhamnolipid increased slightly after incubation for 120 h (Fig 2 1) it is therefore advisable to allow precipitation to occur for 5 days in order to obtain a maximal yield of rhamnolipid

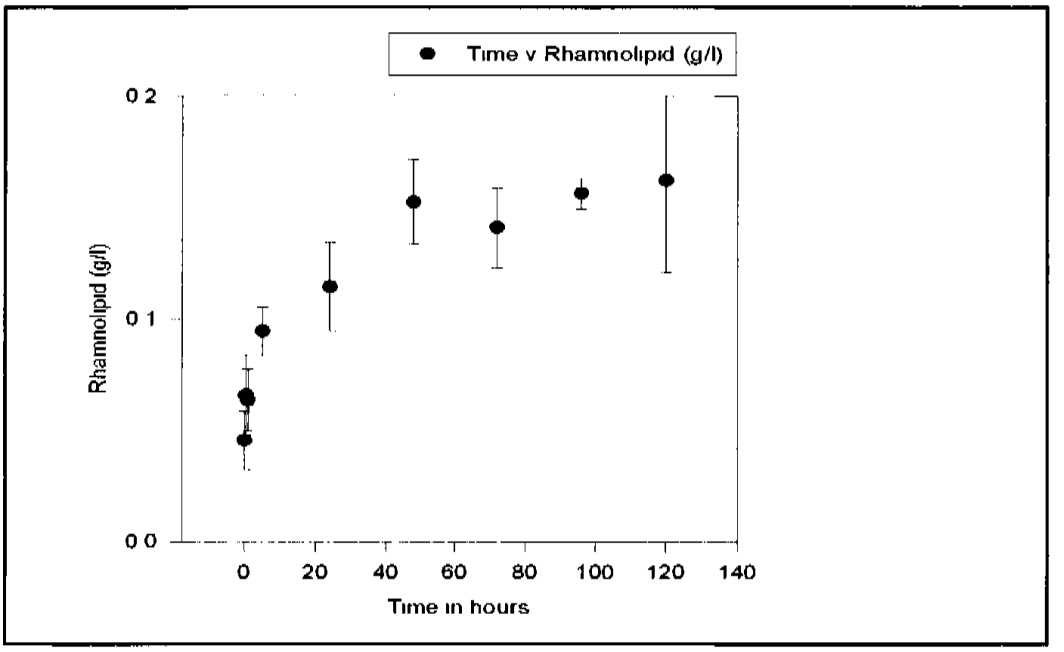


Fig 2 1 Precipitation of rhamnolipid at pH 2 0 4 C for 0 to 120 h Error bars denote one standard deviation of the mean

2 3 3 Extraction of rhamnolipid using different solvents

For the extraction of rhamnolipid from large batch cultures (at least 1 L) the rhamnolipid is often pelleted by centrifugation directly after acidification of the culture supernatant with concentrated HCl (Neilson 1999) Rhamnolipid is then extracted from the pellet which also contains various lipids proteins and sugars Due to the small sample volumes used in this study (1 ml) it was difficult to suspend the small pellet obtained following centrifugation Consequently rhamnolipid was rather extracted directly from the acidified culture supernatant without a centrifugation step

Various different organic solvents were tested for their efficiency in extracting rhamnolipid from the culture supernatant The organic solvents tested were diethyl ether benzene and a mixture of chloroform and methanol Of these benzene did not evaporate readily while extraction with diethyl ether proved to be relatively inefficient compared to the use of a chloroform methanol mixture Extraction of the acidified culture supernatant with chloroform methanol (2 1) resulted in a 36% higher yield of rhamnolipid compared to extraction with diethyl ether Furthermore the chloroform methanol mixture was also less volatile making it safer to work with Although the inclusion of methanol in the solvent mixture is aimed at dissolving the rhamnose sugar moiety of rhamnolipid the high content of methanol in the extraction mixture led to the formation of a thick milky interphase between the organic and aqueous phases during the extraction process and complicated recovery of a clear organic phase As a consequence the OD_{620} readings were not reproducible The milky interphase might have resulted from other saccharides present in the culture medium that settled at the interphase However by adjusting the ratio of chloroform to methanol from 2 1 to 10 1 (Pers comm Neilson 1999) these problems were overcome and reproducible OD_{620} readings could be obtained

In contrast to other rhamnolipid extraction and detection methods which extract the rhamnolipid from batch cultures in separatory funnels (Deziel *et al* 1996 Neilson 1999) extraction of rhamnolipid in this study was performed in glass test tubes from a small sample volume Phase separation of the chloroform methanol mixture and the sample medium occurred very slowly in the test tube and resulted frequently in contamination of the organic phase with cellular sugars thereby compromising the accuracy of the anthrone assay Consequently the time allowed for phase separation was increased from 15 to 45 min This allowed for clear separation of the phases and minimised contamination of the organic phase with cellular sugars As a control the individual components of the PPGAS medium (peptone glucose ammonium salts) were also extracted with the chloroform methanol (10 1) mixture and subjected to the anthrone assay No significant background readings were obtained indicating that the medium constituents did not interfere with the assays and did not compromise the accuracy thereof Despite the small volume of sample used (1 ml) the yield of extracted rhamnolipid (2 93 g/l) extrapolated well to yields of rhamnolipid obtained from batch cultures using other extraction methods (Sim *et al* 1997 Neilson 1999 Deziel *et al* 1996)

2 3 4 Continuous monitoring of rhamnolipid synthesis by *P aeruginosa* PAO1 grown in batch

Having optimised the rhamnolipid assay it was applied to the continuous monitoring of rhamnolipid synthesis by *P aeruginosa* PAO1 grown in batch In addition to determining the glucose and the rhamnolipid concentrations in the growth medium biomass parameters such as cell density (OD_{605}) and the culturable count (CFU/ml) were also determined (Fig 2 2) Similar to previous reports (Ochsner *et al* 1994 Medina *et al* 2003b) synthesis of rhamnolipid by the *P aeruginosa* culture started with the onset of stationary phase and production of rhamnolipid continued up to the sixth day

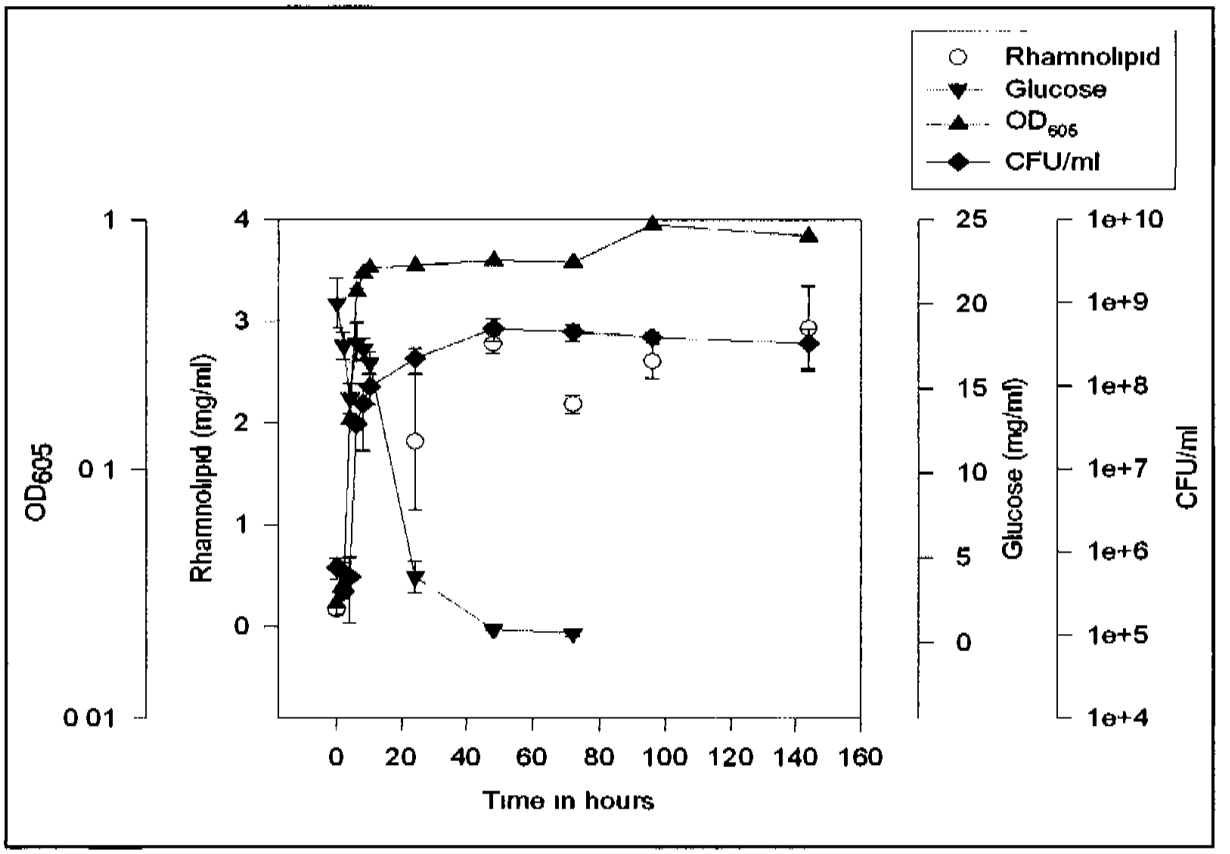


Fig 2 2 Growth related synthesis of rhamnolipid In this assay rhamnolipid yield glucose consumption optical density of the culture and culturable counts were determined Error bars denote one standard deviation of the mean

A slight increase in optical density of the cells was observed after 96 h of culturing and would suggest an increase in pyocyanin production The PPGAS culture medium has two carbon sources proteose peptone and glucose *P aeruginosa* PAO1 used the peptone as carbon source first before

it switched to the glucose from stationary phase onwards This concurs with evidence (Mulligan and Gibbs 1989) showing a direct relationship between the synthesis of rhamnolipid and glutamine synthetase activity in *P aeruginosa* RC II The concentration of D L glutamine L glutamic acid and ammonia in the medium plays an important role where the utilisation of glutamate and ammonia result in the medium becoming more nitrogen limiting thus resulting in further depression of glutamine synthetase Cell metabolism is then regulated to switch from nitrogen based carbon sources to glucose resulting in amongst various other regulatory effects an increase in rhamnolipid production Although the glucose assay indicated an initial glucose concentration of four times higher (20 mg/ml) than the actual concentration (5 mg/ml) the assay gave reproducible results indicating utilisation of glucose upon entering stationary phase The high values could be attributed to the fact that the glucose assay was developed for blood serum and plasma with very low glucose levels

2 4 CONCLUSIONS

The aim of this part of the study was to develop a method whereby rhamnolipid could be extracted from a small sample volume and quantified accurately During the course of developing the protocol various parameters were optimised The most drastic improvement in rhamnolipid yield was obtained by substituting diethyl ether with a chloroform methanol mixture for the extraction of rhamnolipid Contamination of the rhamnolipid containing organic phase with cellular sugars was minimised by allowing the organic and aqueous phases to separate for a longer time (45 min) and this allowed for reproducible measurements of the rhamnolipid concentration Although the rhamnolipid assay described here has the advantage of being simple to perform cost effective and does not rely on the use of specialised equipment it does however have some disadvantages Most notably the assay is not able to distinguish between different rhamnolipids or between different homologs of a specific rhamnolipid Therefore the values are not representative of the actual rhamnolipid concentrations in the culture medium Nevertheless the optimised assay described here was applied to the continuous monitoring of rhamnolipid synthesis by a *P aeruginosa* PAO1 batch culture The assay has been used successfully in this study to screen putative *rhlAB* deficient *P aeruginosa* PAO1 strains (Chapter 3) and it has also been used by a fellow PhD student to characterise rhamnolipid production in biofilms of a RpoS deficient strain of *P aeruginosa*

CHAPTER 3

CONSTRUCTION OF A BROAD-HOST-RANGE MOBILISABLE RECOMBINATION SYSTEM FOR TARGETED INSERTIONAL INACTIVATION OF THE *rhlAB* OPERON IN *Pseudomonas aeruginosa* PAO1

3 1 INTRODUCTION

The construction of mutations in bacteria where a gene is replaced by an *in vitro* modified or an *in vitro* inactivated allele followed by analysis of the resulting effects on the microorganism is frequently used in microbiology research for the identification and study of gene function Four basic methods have been developed to deliver and insert DNA sequences into the chromosomes of microbial cells One method relies on transposons to insert DNA randomly into the chromosome whereas the other three methods facilitate site specific insertions at a gene of interest

Although transposons have been used to randomly insert genes into the bacterial chromosome or to randomly inactivate genes (Hayes 2003) they are not ideally suited for constructing mutant strains with specific targeted mutations This is mainly due to the ability of most natural transposons to catalyse their own movement to alternate sites within a chromosome Furthermore insertion of a transposon into the chromosome may negatively affect expression of downstream genes thus confusing the assignment of a mutant phenotype to the disrupted gene Nevertheless transposon mutagenesis represents a powerful approach towards identifying genes involved in a specific function provided that an appropriate high throughput screen is available

In contrast to transposon mutagenesis allelic exchange methods represent a powerful approach to specifically mutate or inactivate a gene of interest The classical method of allelic exchange relies on the use of so called suicide plasmids that are unable to replicate in the bacterial strain under investigation In such instances a specific DNA fragment is cloned into a plasmid and introduced into a recipient strain where the plasmid cannot replicate Since the plasmid cannot replicate selection for some property on the plasmid such as an antibiotic resistance marker results in isolates that have integrated the plasmid into the bacterial host chromosome via homology between the cloned DNA fragment and the corresponding region of the recipient chromosome Plasmids used as the backbone for such targeted insertional mutagenesis must be conditional for replication in the recipient strain must carry a selectable marker should ideally be transferable to a variety of other bacteria and have an array of unique cloning sites (Goldberg and Ohman 1987) Plasmids that can be transferred by conjugation are preferable due to its higher efficiency compared to transformation or electroporation (Simon *et al* 1986) and the DNA enters the cell in a single stranded form that is more conducive to recombination than double stranded DNA (Alexeyev 1999) This approach is however often inefficient because the frequency of double crossover

events may be low and illegitimate recombination may occur (Toder 1994) Consequently allelic exchange mutants may represent only a small fraction of the transformants

Since double crossover events that incorporate a gene from a plasmid into the chromosome are rare it is not feasible to screen for such events if the cloned gene cannot be directly selected In such cases counter selectable markers may be more useful Under appropriate growth conditions a counter selectable gene promotes the death of the microorganism harbouring it (Gay *et al* 1985) Thus transformants that have integrated a suicide vector containing a counter selectable marker retain a copy of the counter selectable marker in the chromosome and are therefore eliminated in the presence of the counter selectable compound Consequently counter selectable markers have been used for the positive selection of mutants that have undergone targeted genetic alterations together with the loss of the marker (Ried and Collmer 1987 Schweizer 1992 Schweizer and Hoang 1995) A popular counter selectable marker is the *Bacillus subtilis sacB* gene coding for levansucrase In its natural gram positive host range its expression is harmless to the bacterium However cloning of *sacB* in gram negative bacteria leads to the death of the transformed bacteria when grown in the presence of 5% sucrose probably because the accumulation of levans (high molecular weight fructose polymers synthesised by levansucrase) in the periplasm of the gram negative bacteria may be toxic (Steinmentz *et al* 1983 Gay *et al* 1985)

In addition to the above plasmid incompatibility (Inc) has also been exploited as a means to select for marker exchange in various gram negative bacteria (Corbin *et al* 1982) This procedure requires three steps The mutant DNA fragment is firstly cloned into a broad host range plasmid e g a Km^R insertion mutation in a gene may be cloned into a Tc^R pLAH based plasmid (Bertani *et al* 1999) after which the Km^R Tc^R pLAH plasmid is conjugated into the recipient strain by selecting for Tc^R Recipients carrying this pLAH plasmid are then mated with a strain carrying a second IncP1 plasmid with a different selectable marker Plasmid pPH1JI which confers resistance to gentamicin (Gm^R) is often used for this step which is also referred to as the kickout step Since two IncP1 plasmids cannot co exist in the same host cell selection for Gm^R from the pPH1JI plasmid results in loss of the Km^R Tc^R pLAH plasmid However if both Gm^R and Km^R are selected exconjugants arise that have lost the original Tc^R pLAH plasmid and the Km^R marker has integrated into the host chromosome via the homology surrounding the insertion Loss of the pLAH vector can subsequently be confirmed by streaking on tetracycline containing agar plates

Since many of the chromosome integration systems retain the integrated antibiotic resistance markers or other functional plasmid derived DNA that was inserted into the chromosome integration systems have consequently been constructed whereby these sequences can be precisely removed once the desired chromosomal modifications have been constructed Removing antibiotic selection markers and inserted vector derived sequences not only allows for subsequent rounds of mutagenesis/inactivation without accumulating additional resistance markers but also yields modified bacterial strains that are free of DNA sequences that might interfere with subsequent rounds of homologous recombination (Marx and Lidstrom 2002) Examples of such recombination/site excision systems include Flp *FRT* from *Saccharomyces cerevisiae* (Cherepanov and Wackernagel 1995 Hoang *et al* 1998) and Cre *lox* from bacteriophage P1 (Sternberg *et al* 1981 Marx and Lidstrom 2002) Another highly efficient system for site specific recombination aided gene replacement in *P aeruginosa* has been reported (Wong and Mekalanos 2000) *P aeruginosa* was genetically manipulated to express the I *Sce*I restriction endonuclease Following conjugation of a suicide vector containing the replacement gene between two I *Sce*I cleavage sites into the recipient host it is exposed to the I *Sce*I endonuclease and cleaved Cleavage of the DNA prevents the formation of a co integrate and stimulates subsequent homologous recombination Although this method yielded 100% efficiency it is dependant on the use of a genetically manipulated recipient host organism to express the endonuclease Since the endonuclease encoding gene is integrated into a non essential region of the bacterial genome it my be difficult to perform gene replacements in hosts of which the genomic sequences are not yet available

The aim of this part of the investigation was to generate a *rhlAB* deficient *P aeruginosa* PAO1 strain for use in studies whereby the influence of rhamnolipid on the architecture of *P aeruginosa* biofilms could be investigated Although recombinant pUC plasmids have been used by members of our research group as suicide vectors to generate mutant *P aeruginosa* strains it became evident that not all cloned DNA fragments amongst them *rhlAB* could be stably maintained in these plasmids Despite several different chromosome integration systems having been reported as highlighted in the preceding sections none of these were available to us Consequently a need arose for the development of an allelic exchange system for use in *P aeruginosa* Three allelic exchange strategies i e plasmid incompatibility the use of a SacB counter selectable marker and a combination of these approaches were investigated The results that were obtained during the course of these investigations indicated that of the three approaches the latter was most efficient in generating the desired *P aeruginosa* mutant strain by means of double crossover events

3 2 MATERIALS AND METHODS

3 2 1 Bacterial strains and media

The bacterial strains and plasmids used in this part of the study are listed in Table 3 1 *P aeruginosa* and *E coli* strains were maintained at 70 C as glycerol cultures and cultured in LB broth (Sambrook *et al* 1989) at 37 C with shaking at 200 rpm For plasmid DNA selection and maintenance in *E coli* the following concentrations of antibiotics were used 100 µg/ml for ampicillin (Ap) 50 µg/ml for neomycin (Nm) 25 µg/ml for chloramphenicol (Cm) 15 µg/ml for tetracycline (Tc) and 10 µg/ml for gentamicin (Gm) The following antibiotics were used to maintain the plasmid DNA and chromosomal insertions in *P aeruginosa* strains and for plasmid DNA selection carbenicillin (Cb) at 250 µg/ml neomycin (Nm) at 200 µg/ml tetracycline (Tc) at 100 µg/ml and gentamicin (Gm) at 100 µg/ml

3 2 2 Genomic DNA isolation

Genomic DNA was isolated from wild type and mutant *P aeruginosa* strains according to the method described by Wilson (1990) with the following modifications The cells from an overnight culture (1 5 ml) were pelleted at 13 000 x *g* for 2 min and suspended in 567 µl of 1 x TE buffer (10 mM Tris HCl 1 mM EDTA pH 8 0) After addition of 30 µl of lysozyme (50 mg/ml) and 50 µl of 10% (w/v) EDTA the suspension was incubated for 1 h at 4 C followed by the addition of 3 µl of Proteinase K (20 mg/ml) and 30 µl of 10% (w/v) SDS Following incubation for 1 h at 37 C 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution (10% [w/v] cetyltrimethylammonium bromide [CTAB] in 0 7 M NaCl) were added and incubation was continued for 10 min at 65 C The suspension was extracted with an equal volume of chloroform isoamyl alcohol (24 1) followed by centrifugation for 5 min at 13 000 x *g* The supernatant was transferred to a clean microfuge tube The residual CTAB was removed by addition of an equal volume of phenol chloroform isoamyl alcohol (25 24 1) followed by centrifugation for 5 min at 13 000 x *g* The genomic DNA was precipitated from the recovered aqueous supernatant by addition of 0 6 volume isopropanol The precipitated genomic DNA was pelleted by centrifugation for 2 min at 13 000 x *g* washed with 70% ethanol dried under vacuum and suspended in 80 µl 1 x TE buffer or ddH_2O An aliquot (2 µl) of the genomic DNA was analysed by electrophoresis on a 0 8% (w/v) agarose gel

Table 3 1 Bacterial strains plasmids and primers used in this study

		Relevant genotype or phenotype	Source or reference
Strains			
P aeruginosa	PAO1	Wild type	Holloway *et al* (1979)
	SS1258	PAO1 Δ*rhlAB*004 *tet*^{R}	Suh (2002)
	PAO ABK6	PAO1 Δ*rhlAB kan*^{R}	This study
E coli	DH5α	F *recA1 endA1 hsdR17 deoR thi 1 supE44 gyrA96 relA1* Δ(*lacZYA argF*) U169 λ [Φ80d*lacZ*ΔM15]	Sambrook *et al* (1989)
Plasmids			
	pFLP2	Ap^R *ori1600 oriT* source of *sacB* 9 3 kb	Hoang *et al* (1998)
	pJB3Tc20	Ap^R Tc^R RK2 replicon ColE1 replicon *oriT* polylinker and alpha complementation region from pUC119 IncP 7 1 kb	Blatny *et al* (1997)
	pJBSac	Derivative of pJB3Tc20 in which a 3 kb *Eco*RV fragment containing *sacB* from pFLP2 was cloned into the *Eco*RV site in *tetA* Ap^R 10 1 kb	This study
	pLAH31	Tc^R IncP1 broad host range *oriT* contains a His tag expression cassette 22 kb	Bertani *et al* (1999)
	pLAH AB	Derivative of pLAH31 where the digested PCR product (*Bgl*II *Hin*dIII) of *rhlAB* was cloned into *Bam*HI/*Hin*dIII of the multiple cloning site 24 5 kb	This study
	pLAH ABK	Derivative of pLAH AB where an internal 1 kb fragment from *rhlAB* was removed with *Pst*I and replaced with a 1 3 kb Km^R cassette from pUC4K Tc^R Km^R 25 kb	This study
	pPH1JI	Sm^R Gm^R IncP *ori*T 60 kb Used as a chase plasmid	Beringer *et al* (1978)
	pPT212	Gm^R IncP RK2 replicon polylinker and alpha complementation region from pUC118 *rpoN* fragment (10 2 kb) in *Bam*HI *oriT* 18 kb	Totten *et al* (1990)
	pPT212S	Derivative of pPT212 where the 10 2 kb *rpoN* fragment was removed with *Bam*HI and plasmid re ligated 8kb	This study
	pPTSac	Derivative of pPT212S where a 2 6 kb *Xba*I/*Hin*dIII fragment from pFLP2 containing *sacB* was cloned into the *Xba*I/*Hin*dIII sites in the polylinker 10 5 kb	This study
	pPTS ABK	Derivative of pPTSac where the blunt ended PCR product *rhlAB* Km^R (2 4 kb) from pLAH ABK was cloned into *Sma*I of the polylinker 13 kb	This study
	pRK600	Cm^R ColE1 replicon RK2 Mob RK2 Tra helper plasmid in triparental conjugations	Kessler *et al* (1992)
	pUC4K	Ap^R Km^R pUC based vector with *kan* cassette 4 kb	Vieira and Messing (1989)

		Relevant genotype or phenotype	Source or reference
	pGEM® T Easy	Ap^R ColE1 replicon TA cloning vector for PCR products	Promega
Primers			
	ForBgl	5 GGGAAGATCTTGAAATGCGGCGCGAAAGTC 3	This study
	RevHind	5 CCCAAGCTTCGGCAAAATCATGGCAACCC 3	This study
	FFP1	5 TGAAATGCGGCGCGAAAGTC 3	This study
	MRP	5 CGGCAAAATCATGGCAACCC 3	This study

Underlined is the recognition sequence for *Bgl*II in primer ForBgl and for *Hind*III in primer Re Hind Both restriction sites are stabilised with G or C clamping sequences

3 2 3 Polymerase chain reaction (PCR)

3 2 3 1 Design of oligonucleotide primers

The oligonucleotide primers used in PCRs to amplify *rhlAB* from the extracted *P aeruginosa* genomic DNA are indicated in Table 3 1 Since the amplicon was to be cloned into the expression vector pLAH31 the upstream primers were designed so as to exclude the ribosome binding site but include the start codon (ATG) The primers were designed based on the published *P aeruginosa* PAO1 genome sequence (Stover *et al* 2000) available at http //www pseudomonas com using DNAMAN V 4 13 (Lynnon Biosoft) and Amplify 1 2 software programs Unique restriction enzyme recognition sequences were incorporated at the 5 terminus of primers RevHind and ForBgl following restriction analysis of the target DNA using BioEdit Sequence Alignment Editor V 5 0 9 (Hall 1999) BLASTN available at http //www ncbi nlm nih gov/BLAST was used to verify the target sequence specificity of the newly designed primer pair The primers were synthesised by Integrated DNA Technologies Inc

3 2 3 2 PCR amplification

The target DNA was amplified either with SUPERTHERM DNA polymerase or *Pwo* DNA polymerase thereby necessitating the use of different reaction conditions as indicated below For PCR amplification of the target DNA using SUPERTHERM DNA polymerase the reaction mixtures (50 µl) contained template DNA (200 ng) 200 µM of each dNTP 30 pmol primer ForBgl 34 pmol primer RevHind 1 U of SUPERTHERM DNA polymerase (Southern Cross Biotechnology) 5% (v/v) DMSO 2 5 mM $MgCl_2$ and 1 x PCR buffer (as supplied) Following incubation at 94 C for 3 min the samples were subjected to 35 of the following cycles using an

Applied Biosystems GeneAmp® 2400 thermal cycler denaturation for 30 s at 94 C annealing for 45 s at 55 C and elongation for 2 min at 72 C followed by elongation for 5 min at 72 C in the final cycle Alternatively *Pwo* DNA polymerase (Roche) which possesses a highly processive 5 to 3 polymerase activity and a 3 to 5 exonuclease activity was used to generate blunt ended PCR products for blunt end ligations The first reaction mixture (25 µl) contained 0 5 µg of template DNA (pLAH ABK) 200 µM of each dNTP 60 pmol of primer ForBgl 60 pmol of primer RevHind and ddH_2O The second reaction mixture (25 µl) contained 1 x PCR buffer (10 mM Tris HCl pH 8 85 25 mM KCl 5 mM $(NH_4)_2SO_4$ 2 mM $MgSO_4$) 2 5 U of *Pwo* DNA polymerase and ddH_2O Following a hot start the reaction mixtures were mixed and subjected to 35 of the following cycles in a Applied Biosystems GeneAmp® 2700 thermal cycler denaturation at 94 C for 15 s annealing at 55 C for 30 s elongation at 72 C for 2 min with an increase of 5 s in all subsequent elongation cycles followed by elongation for 7 min at 72 C in the final cycle

3 2 4 Agarose gel electrophoresis

DNA was analysed on 0 8% (w/v) agarose gels supplemented with 1 µg/ml ethidium bromide (Sambrook *et al* 1989) The agarose gels were electrophoresed at 90 V in 1 x TAE buffer (40 mM Tris HCl 2 mM EDTA 20 mM acetic acid pH 8 0) and the DNA visualised on a Vilber Lourmat transilluminator Gel images were captured with a digital gel documentation system (Vilber Lourmat)

3 2 5 Isolation of DNA fragments from agarose gels

DNA fragments were purified from agarose gel slices as described by Boyle and Lew (1995) The DNA fragments were excised from the agarose gel with a scalpel blade mixed with 1 ml of a 6 M NaI solution per 1 g of agarose gel and the agarose was melted by incubation at 55 C Following complete dissolution of the agarose 10 µl of a silica suspension was added to the sample vortexed for 10 s and then incubated at 4 C for 30 min with intermittent vortexing The silica bound DNA was pelleted by centrifugation for 30 s at 13 000 x *g* and the pellet washed four times with 500 µl ice cold New Wash (50 mM NaCl 10 mM Tris HCl 25 mM EDTA and 50% [v/v] ethanol) The DNA was eluted from the silica matrix at 55 C for 10 min in a final volume of 25 µl of ddH_2O An aliquot (2 µl) of the eluted DNA was analysed by electrophoresis on a 0 8% (w/v) agarose gel to assess the concentration and purity of the DNA

3 2 6 DNA molecular weight markers

Four DNA molecular weight markers were used throughout the study Excepting for the GeneRuler™ 100 bp DNA ladder Plus which was obtained from Fermentas AB the other three DNA molecular weight markers were prepared by digestion of either λ DNA (Roche) or plasmid DNA with different restriction endonucleases Digestion of phage λ DNA with *Hind*III yielded fragments corresponding in size to 23 130 bp 9 416 bp 6 557 bp 4 361 bp 2 322 bp 2 027 bp and 564 bp Alternatively the phage λ DNA was digested with both *Eco*RI and *Hind*III to yield fragments of 21 226 bp 5 148 bp 4 973 bp 4 268 bp 3 530 bp 2 027 bp 1 904 bp 1 584 bp 1 375 bp 947 bp 831 bp and 564 bp The third DNA molecular weight marker was prepared by digestion of plasmid pFLP2 (Hoang *et al* 1998) with both *Bam*HI and *Hind*III to yield DNA fragments of 4 634 bp 2 623 bp 1 310 bp 605 bp and 125 bp All DNA markers were stored at 4 C

3 2 7 Restriction endonuclease digestions

Approximately 200 – 800 ng of plasmid DNA was digested with 2 U of enzyme in the appropriate concentration of salt using the 10x buffer supplied by the manufacturer The reaction volumes were typically 20 – 30 µl and incubation was at 37 C for at least 1 h excepting for high molecular weight plasmid DNA which was incubated overnight For digestions involving two enzymes of which the salt concentrations differed for optimal activity the plasmid DNA was first digested with the enzyme requiring the lowest salt concentration The salt concentration was then adjusted and the second enzyme added All restriction enzymes were supplied by Roche or New England Biolabs Following digestion the DNA fragments of interest were either purified from an agarose gel (Section 3 2 5) or the DNA (e g amplicons) was precipitated from the reaction mixture Briefly 0 3 volume of 7 5 M NH_4OAc and 1 volume of isopropanol was added and incubated at 4 C for 1 h or overnight The precipitated DNA was pelleted by centrifugation at 12 000 x *g* for 10 min rinsed with 70% ethanol dried under vacuum and suspended in an appropriate volume of ddH_2O

3 2 8 Ligation reactions

Ligation reactions were performed in 1 5 ml microfuge tubes and incubated overnight at 16 C in a circulating water bath Insert and vector DNA with a ratio in excess of 3 1 were typically ligated in a 15 µl reaction volume containing 1 x ligation buffer (2 mM Tris HCl 0 1 mM EDTA 0 5 mM DTT 6 mM KCl 5% [v/v] glycerol pH 7 5) and 1 U T4 Ligase (Roche) For cloning of PCR amplicons the pGEM® T Easy vector system (Promega) was used The molar ratio of insert to

vector DNA was determined according to the manufacturer's instructions. The ligation reaction mixtures contained 5 µl of a 2 x Rapid Ligation Buffer, 50 ng of pGEM® T Easy vector DNA, approximately three fold higher molar concentration of purified amplicon, 1 µl T4 DNA Ligase (3 Weiss units/µl) and deionised water to a final volume of 10 µl. Ligation reactions were incubated overnight at 16 °C. Controls consisting of self ligated vector DNA were also included to determine the yield of background non-recombinant clones.

3.2.9 Preparation and transformation of competent *E. coli* DH5α cells

Competent *E. coli* DH5α cells were prepared and transformed according to the method of Inoue as described by Sambrook and Russel (2001). A starter culture was prepared by inoculating a single colony from a freshly streaked culture of *E. coli* DH5α into 25 ml of SOB broth (2% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.05% [w/v] NaCl, 2.5 ml of 1 M KCl, pH 7.0, supplemented with 10 ml sterile 1 M $MgCl_2$ after autoclaving). Following incubation for 6 – 8 h at 37 °C with vigorous shaking (250 – 300 rpm), three 1 L Erlenmeyer flasks containing 250 ml of SOB broth were inoculated with 10 ml, 4 ml and 2 ml of the starter culture respectively. The cultures were incubated overnight at 18 – 22 °C with moderate shaking, after which the OD_{600} was measured every 45 min. Once an OD_{600} of 0.55 was reached, the flasks were transferred to an ice water bath for 10 min to inhibit further growth. The cells were then harvested by centrifugation at 2 500 x *g* for 10 min at 4 °C. The supernatant was discarded and the tubes were placed upside down on paper towels for 2 min to drain away excess fluid. The cell pellets were suspended by gentle swirling in 80 ml of filter sterilised ice cold transformation buffer (55 mM $MnCl_2 4H_2O$, 15 mM $CaCl_2 2H_2O$, 250 mM KCl, 10 mM PIPES [pH 6.7]). The cells were harvested (2 500 x *g*, 10 min, 4 °C) and then suspended in 20 ml of the transformation buffer. Following addition of 1.5 ml of DMSO and incubation on ice for 10 min, the cells were aliquoted in 1.5 ml microfuge tubes and snap frozen in liquid nitrogen prior to storage at -70 °C.

Prior to transformation, the competent cells were thawed on ice for 10 min. The cells and ligation reaction mixture (not more than 5% of the volume of the competent cells) were mixed and incubated on ice for 30 min. After a heat shock at 42 °C for 90 s in a pre-heated circulating water bath, the tubes were placed immediately on ice for 2 min. Subsequently, 800 µl of SOC broth (identical to SOB except for the addition of 20 ml of sterile 1 M glucose prior to use) was added and the cells incubated for 45 min at 37 °C before plating aliquots of 100 µl on selective agar plates. Where transformants were selected based on the tetracycline resistance marker, the cells were first

centrifuged for 2 min at 13 000 x *g* and then resuspended in 100 µl of sterile SOC broth before plating the total volume For cloning procedures involving pGEM® T Easy the cells were plated on LB agar plates containing ampicillin X Gal (40 µg/ml) (Roche) and IPTG (0 2 mM) (Roche) for blue white selection As a control competent cells alone were plated on the selective agar plates to check for contamination of the competent cells

3 2 10 Plasmid DNA isolation

Plasmid DNAs were extracted according to a modified alkaline lysis method (Sambrook *et al* 1989) Single colonies were each inoculated into 5 ml LB broth supplemented with the appropriate antibiotic and cultured at 37 C with shaking The cells from 1 5 ml of the overnight culture were harvested by centrifugation for 2 min at 13 000 x *g* in a 1 5 ml microfuge tube The cell pellet was suspended in 100 µl of Solution 1 (50 mM Tris HCl 10 mM EDTA pH 8 0) and incubated at room temperature for 5 min followed by 1 min on ice The cells were lysed by the addition of 200 µl Solution 2 (200 mM NaOH 1% [v/v] SDS) and after incubation on ice for 5 min 300 µl of Solution 3 (2 80 M KAc pH 5 1) was added After incubation on ice for 10 min the insoluble aggregate that formed was collected by centrifugation at 13 000 x *g* for 15 min The plasmid DNA was precipitated from the recovered supernatant by addition of 800 µl ice cold 96% ethanol and incubation at 70 C for 30 min The DNA was pelleted by centrifugation at 13 000 x *g* for 15 min rinsed with 70% ethanol dried under vacuum and suspended in 20 – 30 µl ddH_2O To remove contaminating RNA extracted plasmid DNA was incubated with 1 µl RNase A (10 mg/ml) for 30 min at 37 C

A commercial DNA extraction and purification kit (NUCLEOBOND® AX100 Macherey – Nagel GmbH and Co) was used for isolation of cosmid pLAH31 according to the manufacturer s instructions Briefly the cells from 100 ml of an overnight culture (37 C 200 rpm) were collected by centrifugation at 3 000 x *g* for 10 min at 4 C The cell pellet was suspended in 8 ml buffer S1 (50 mM Tris HCl 10 mM EDTA 100 µg/ml RNaseA pH 8 0) after which 8 ml buffer S2 (200 mM NaOH 1% [v/v] SDS) was added and the suspension incubated at room temperature for 5 min To this 8 ml buffer S3 (2 80 M KAc pH 5 1) was added and incubation was continued on ice for 5 min Following centrifugation at 12 000 x *g* for 25 min at 4 C the supernatant was carefully recovered and loaded onto a NUCLEOBOND® cartridge pre equilibrated with 2 5 ml buffer N2 (100 mM Tris/H_3PO_4 15% ethanol 0 15% Triton X 100 0 9 mM KCl pH 6 3) The flow through was collected and reloaded onto the cartridge The cartridge was subsequently washed three times

with 4 ml buffer N3 (100 mM Tris/H_3PO_4 15% ethanol 1 15 M KCl pH 6 3) after which the plasmid DNA was eluted with pre warmed (50 C) buffer N5 (100 mM Tris/H_3PO_4 15% ethanol 1 M KCl pH 8 5) The purified plasmid DNA was precipitated by addition of 0 8 volume of isopropanol and collected by centrifugation for 30 min at 13 000 x *g* at 4 C The DNA pellet was rinsed with 70% ethanol dried at room temperature and suspended in ddH_2O

3 2 11 Nucleic acid sequencing

Nucleotide sequencing of cloned insert DNA was performed using the BigDye™ Terminator v3 1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems) The sequencing reactions contained 2 µl Ready Reaction mix 1 µl sequencing primer (3 2 pmol/µl) (Table 3 1) 100 ng of gel purified template DNA and ddH_2O to a final volume of 10 µl Cycle sequencing reactions were performed in an Applied Biosystems GeneAmp® 2700 thermal cycler with 25 of the following cycles denaturation at 96 C for 10 s primer annealing at 50 C for 5 s and elongation at 60 C for 4 min The extension products were precipitated by the addition of 4 µl ddH_2O and 16 µl 96% ethanol to each of the sequencing reactions The tubes were vortexed briefly incubated at room temperature for 30 min in the dark centrifuged at 13 000 x *g* for 30 min and the supernatant carefully aspirated The pellets were rinsed with 50 µl 70% ethanol vacuum dried and stored at 20 C Prior to electrophoresis the purified extension products were suspended in 3 5 µl Blue dextran/EDTA loading buffer denatured for 2 min at 90 C and loaded onto either an ABI PRISM 377 DNA Sequencer or an ABI PRISM 3100 Genetic Analyzer Nucleotide sequences were edited using BioEdit Sequence Alignment Editor V5 0 9 (Hall 1999) and their identity verified by BLASTN searches against the GenBank database at www ncbi nlm nih gov/BLAST as well as the *P aeruginosa* genome sequence at www pseudomonas com

3 2 12 Plasmid construction

All molecular cloning techniques employed in the construction of the respective vectors were performed according to the procedures described in the preceding sections All plasmid constructs were confirmed by restriction endonuclease digestions or by PCR analysis

pLAH AB

Primers ForBgl and RevHind were used with genomic DNA from *P aeruginosa* PAO1 to generate a 2 3 kb product containing the *rhlAB* genes (Section 3 2 3 2) The amplicon was digested with both *Bgl*II and *Hind*III and then cloned into identically digested pLAH31 yielding pLAH AB

- **pLAH-ABK**

Recombinant plasmid pLAH-AB was digested with *Pst*I to remove a 1-kb DNA fragment containing 300 bp of the *rhlA* ORF and 600 bp of the *rhlB* ORF. A 1.2-kb kanamycin resistance cassette was recovered from pUC4K by *Pst*I digestion and then ligated into the deletion site of pLAH-AB to generate the allelic exchange vector pLAH-ABK (Fig. 3.1).

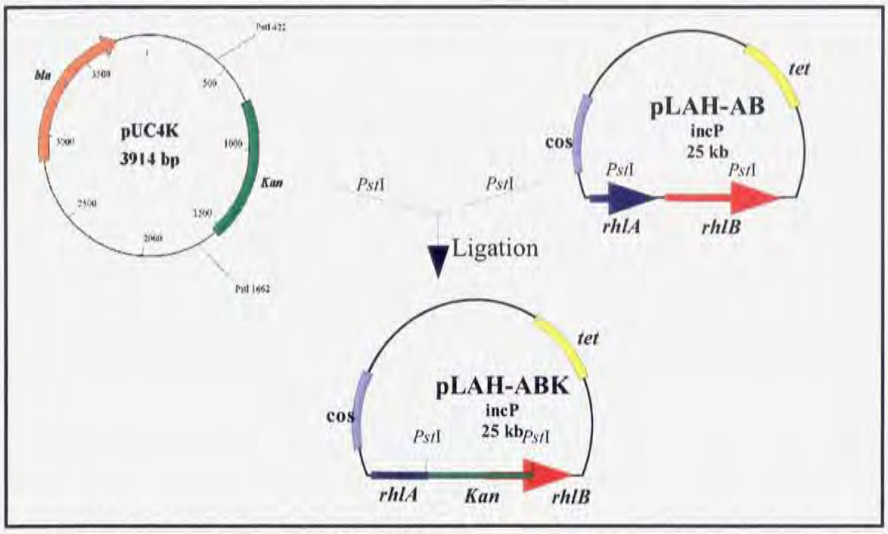


Fig. 3.1 Strategy used for the construction of pLAH-ABK.

- **pPTSac**

Recombinant plasmid pPT212 was digested with *Bam*HI to excise a previously cloned copy of *rpoN*. The plasmid DNA was gel-purified and self-ligated to yield plasmid pPT212S. To construct pPTSac, plasmid pPT212S was digested with both *Xba*I and *Hind*III and ligated with the 2.6-kb *sacB* counter-selectable marker, which had been recovered from pFLP2 by *Xba*I and *Hind*III digestion (Fig. 3.2).

- **pPTS-ABK**

A 2.4-kb *rhlAB*::*kan*^{R} amplicon, obtained by PCR amplification using pLAH-ABK plasmid DNA as template and *Pwo* DNA polymerase, was inserted by blunt-end ligation into the *Sma*I site of plasmid pPTSac, thereby completing the construction of the allelic exchange vector pPTS-ABK.

- **pJBSac**

Plasmid pFLP2 was digested with *Eco*RV to excise a 3-kb DNA fragment containing the *sacB* counter-selectable marker and then cloned into *Eco*RV-digested plasmid pJB3Tc20 to generate pJBSac (Fig. 3.3).

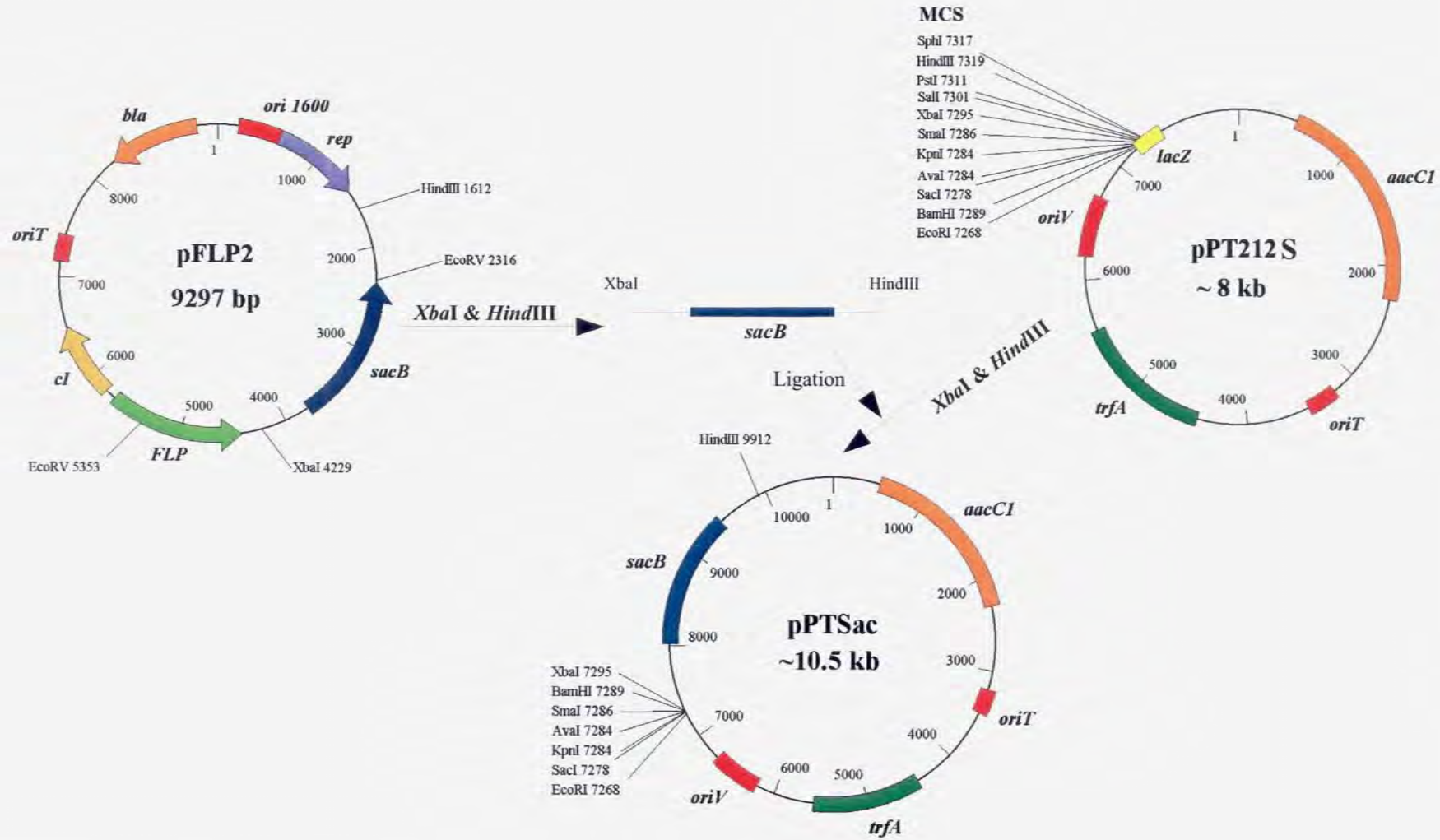


Fig. 3.2 Strategy used for the construction of pPTSac (See text for details).

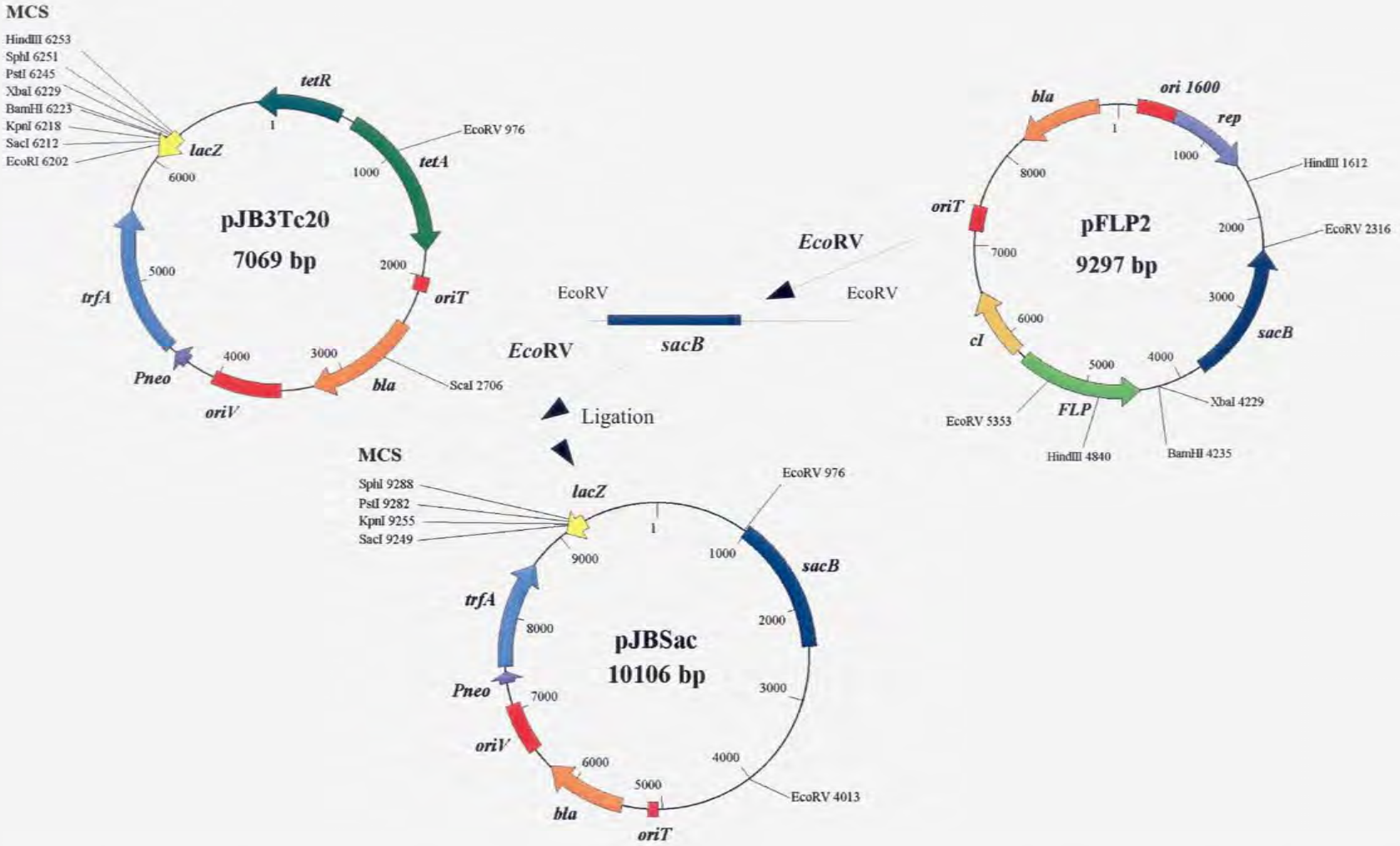


Fig. 3.3 Strategy used for the construction of pJBSac (see text for details). As a consequence of the cloning strategy, the DNA sequences flanking the *sacB* gene contain restriction enzyme recognition sites contained in the multiple cloning site of pJB3Tc20. Thus, only unique recognition sites are indicated in the multiple cloning site of pJBSac. The orientation of the cloned *sacB* gene was not determined.

3 2 13 Generation of tri transconjugants

Plasmids were introduced into *P aeruginosa* PAO1 by triparental conjugation (Figurski and Helinski 1979) Triparental matings were performed with helper plasmid pRK600 as previously described (Kessler *et al* 1992) A single colony of freshly streaked cultures of donor (*E coli* DH5α containing the donor plasmid) recipient (*P aeruginosa* PAO1) and helper (*E coli* DH5α containing pRK600) strains were mixed on a LB agar plate with a sterile inoculation loop and then incubated overnight at 37 C Donor recipient and helper strains were mixed in volumetric ratios of 2 1 1 on as small an area as possible to improve contact between the respective bacterial strains Following incubation the mixed growth was streaked on selective LB agar plates containing the appropriate antibiotics and incubated for 24 to 48 h at 37 C Successful transfer of the plasmid DNA to *P aeruginosa* was confirmed by re streaking on a LB agar plate supplemented with the appropriate antibiotics

3 2 14 Southern blot analysis

Southern blot analysis was performed to confirm replacement of the wild type *rhlAB* with the mutant null allele in the putative *P aeruginosa* mutant strains For this purpose the PCR amplified 2 3 kb *rhlAB* amplicon and the pPTSac plasmid backbone were used as probes The probes were prepared and detected using the DIG High Prime kit (Roche)

3 2 14 1 Preparation of labelled rhlAB and pPTSac DNA probes

The 2 3 kb *rhlAB* amplicon was obtained by PCR amplification using genomic DNA of *P aeruginosa* PAO1 as template DNA and primers ForBgl and RevHind as described in Section 3 2 3 Plasmid pPTSac was isolated as described in Section 3 2 10 and digested with *Bsr*BI prior to preparation of labelled probe DNA To prepare the DIG dUTP labelled probes 1 µg of the purified *rhlAB* amplicon and digested pPTSac plasmid DNA was diluted in ddH_2O to a final volume of 16 µl and then denatured by heating in a boiling water bath for 10 min The denatured DNA was then immediately chilled in an ice water bath and mixed with 4 µl DIG High Prime (consisting of DIG dUTP Klenow polymerase dNTPs buffer and random primers) Following overnight incubation at 37 C the reactions were terminated by the addition of 2 µl 0 2 M EDTA (pH 8 0) The concentration and labelling efficiency of the probe DNA were subsequently determined using the control labelled DNA supplied with the kit according to the manufacturer s instructions The probes were used at a final concentration of 20 ng/ml and required that 6 25 µl of the labelled *rhlAB* and 12 5 µl of the labelled pPTSac probe be used in 25 ml of hybridisation solution

3 2 14 2 Preparation of the membrane

For hybridisation using the labelled *rhlAB* probe preparations of the genomic DNA of *P aeruginosa* wild type PAO1 and putative *rhlAB* mutant strains (Section 3 2 2) were digested with *Pst*I overnight at 37 C (4 U of *Pst*I per 2 µg of genomic DNA) Genomic DNA of *E coli* as well as plasmids pJB3Tc20 and pPTSac were included in the analysis as negative controls For hybridisation using the labelled pPTSac probe the preparations of genomic DNA were digested with *Ava*I overnight at 37 C (4 U of *Ava*I per 2 µg of genomic DNA) pPTSac plasmid DNA was included in the analysis as positive control to confirm probe specificity The genomic and plasmid DNA samples were separated by electrophoresis on a 1% (w/v) agarose gel (Section 3 2 4) and then transferred from the agarose gel to a Hybond™ N (Amersham Life Science) nylon membrane by capillary blotting For this purpose the DNA was first denatured by soaking the gel for 45 min with constant agitation in denaturing solution (1 5 M NaCl 0 5 M NaOH) rinsed in ddH_2O and then neutralised as above in neutralisation solution (1 M Tris HCl [pH 7 2] 1 5 M NaCl 10 mM EDTA) Two pieces of filter paper soaked in 20 x SSC (3 M NaCl 0 3 M sodium citrate pH 7 0) were stacked on a piece of Glad Wrap™ folded so as to surround the gel The inverted gel (with the orientation marked by cutting one corner) was placed onto the filter paper followed by the nylon membrane and then 2 pieces of filter paper all of which were pre wet in 2 x SSC Four additional dry filter papers and several paper towels were stacked on top of the wet filter papers and weighed down by a light weight Transfer was allowed to proceed at room temperature for at least 18 h after which the membrane was rinsed in 2 x SSC and the DNA fixed to the membrane by UV irradiation for 5 min each side

3 2 14 3 Hybridisation

The hybridisation temperature was calculated using the following formula (Roche)

T_m = 49 82 + 0 41(%GC) – (600/*l*) where *l* is the length of the hybrid in bp The optimum hybridisation temperature was then calculated using $T_{pt} = T_m - 25$ C The optimal hybridisation temperature for the *rhlAB* probe was calculated at 50 C and for the pPTSac probe at 45 7 C The membranes were incubated in the DIG Easy Hyb buffer pre warmed to the appropriate temperature and pre hybridised for 30 min with gentle agitation The pre hybridisation buffer was then decanted and replaced with 25 ml of hybridisation buffer so as to cover the membrane after which 20 ng/ml of the denatured labelled *rhlAB* or pPTSac probe DNA was added to the buffer The probe DNA was denatured by heating in a boiling water bath for 5 min and placed immediately on ice prior to being added to the membrane Hybridisation was allowed to proceed overnight at 50 C (for the

rhlAB probe) and 45 7 C (for the pPTSac probe) After hybridisation the membranes were washed twice for 5 min in 2 x SSC 0 1% (v/v) SDS at 68 C

3 2 14 4 Detection of hybridised probe DNA

The hybridised probes were detected by rinsing the membranes for 5 min in washing buffer (0 1 M Maleic acid 0 15 M NaCl [pH 7 5] 0 3% [v/v] Tween 20) followed by incubation for 30 min at room temperature in 1 x blocking solution (prepared by diluting the supplied blocking solution 10 fold in Maleic acid buffer [0 1 M Maleic acid 0 15 M NaCl pH7 5]) The membranes were then incubated for 30 min at room temperature with gentle agitation in 20 ml of the anti digoxigenin alkaline phosphatase conjugated antibody solution (diluted 1 5000 [150 mU/ml] in blocking solution) The unbound antibodies were removed by washing the membrane twice for 15 min each wash in washing buffer at room temperature with gentle agitation and equilibrated for 3 min in detection buffer (0 1 M Tris HCl 0 1 M NaCl pH 9 5) The membranes were then immersed in 10 ml of the enzyme substrate solution (NBT/BCIP stock diluted 1 50 in detection buffer) Once the bands became visible the colour reaction was stopped by adding TE buffer to the solution

3 2 15 Rhamnolipid assay

The putative double crossover mutants were assayed for rhamnolipid synthesis using the optimised rhamnolipid extraction and quantification assay developed previously (Chapter 2) *P aeruginosa* PAO1 was included as a positive control and *P aeruginosa* SS1258 as a negative control (Table 3 1) Following incubation overnight of freshly streaked cultures of the different strains to be assayed a single colony was inoculated into 5 ml of PPGAS medium in triplicate The cultures were incubated for 3 days at 37 C with shaking aeration at 150 rpm after which the rhamnolipid was extracted and precipitated as follows The cells from 1 ml of each culture were pelleted by centrifugation for 5 min at 13 000 x *g* and the supernatant transferred to a new 1 5 ml microfuge tube The rhamnolipid was precipitated after acidifying the supernatant to pH 2 through the addition of 100 µl of concentrated HCl (33%) and incubation at 4 C for 5 days The suspension was subsequently transferred to a 5 ml glass test tube and extracted twice with twice the volume (2 ml) chloroform methanol (10 1) The organic (chloroform) phase was transferred to a new 5 ml glass test tube and the extraction process repeated on the aqueous phase The test tubes containing the chloroform phase were incubated in a water bath at 80 C until the chloroform had evaporated and a thick honey like solution remained at the bottom of the test tube The rhamnolipid was then dissolved in 2 ml of 0 05 M sodium bicarbonate (pH 8 6) vortexed briefly and incubated at room

temperature for at least 30 min The rhamnolipid was quantified using the anthrone assay Briefly samples were overlayed with twice the volume of anthrone solution by slowly adding the reagent to the sample in glass test tubes on ice The test tubes were vortexed boiled in a water bath for 15 min (Sim *et al* 1997) and then allowed to cool to room temperature An aliquot (200 µl) was pipetted into a flat bottom microtitre plate (Greiner) and the absorbency was measured within 1 h at 620 nm against distilled water on a Thermo Labsystems Multiskan Ascent microtitre plate reader

3 3 RESULTS AND DISCUSSION

3 3 1 Generation of *rhlAB* deficient *P aeruginosa* PAO1 strains based on the use of plasmids belonging to the same incompatibility group

Different strategies were used to generate isogenic mutant strains of the wild type *P aeruginosa* PAO1 strain in which the *rhlAB* open reading frames (ORFs) on the *P aeruginosa* genome were replaced with *in vitro* modified alleles In the first of these strategies mutant *P aeruginosa* strains were generated based on the inability of plasmids belonging to the same incompatibility (Inc) group to co exist in the same host bacterium For this purpose an allelic exchange vector was constructed in which the *rhlAB* ORFs were disrupted by the insertion of a cassette encoding kanamycin resistance (*kan*^{R}) Mutants of *P aeruginosa* PAO1 were generated by introducing the allelic exchange vector (pLAH ABK) into the wild type strain followed by the introduction of a second incompatible plasmid (pPH1JI) carrying a gentamicin resistance marker (Gm^R) Subsequent homologous recombination events between the *rhlAB* DNA flanking the *kan*^{R} cassette in the vector and the wild type locus on the genome were selected for by growth in the presence of both Nm^R and Gm^R thus resulting in the loss of the allelic exchange vector backbone

3 3 1 1 Construction of allelic exchange vector pLAH-ABK

3 3 1 1 1 PCR and cloning of the rhlAB genes

To obtain the 2 3 kb *rhlAB* genes oligonucleotide primers FFP1 and MRP (Table 3 1) were designed based on the published sequence of the *P aeruginosa* PAO1 genome (Stover *et al* 2000) Initial attempts at amplifying the *rhlAB* genes were unsuccessful resulting in primer dimer amplification artifacts and/or an amplicon of 500 bp (Fig 3 4 lane 2) *In silico* analysis of the predicted secondary structure of the target region (at 72 C) using MFOLD (Zucker *et al* 1999)

indicated a complex yet stable folding pattern that consisted of numerous hairpin and stem loop structures (Fig 3 5) The complex secondary structure together with the high G+C content of the targeted region (67%) may have prevented efficient annealing of the primers to their intended target sequence or alternatively caused premature detachment of the DNA polymerase from the template DNA during the elongation step of the PCR Since the *rhlAB* region appeared to be refractory to amplification despite numerous optimisation attempts it was investigated whether the addition of an additive such as dimethyl sulfoxide (DMSO) to the reaction mixtures could facilitate PCR amplification of the intended target region DMSO disrupts DNA base pairing and in addition to minimising secondary structures also alters the melting characteristics of the DNA template Addition of DMSO to a final concentration of 5% (v/v) in the PCR reaction mixtures resulted in the amplification of a 2 3 kb DNA fragment which corresponded to the expected size of *rhlAB* (Fig 3 4 lane 3)

The 2 3 kb amplicon was subsequently purified from agarose gel and cloned into the pGEM® T Easy cloning vector Following transformation of competent *E coli* DH5α cells recombinant transformants with a *lac* phenotype were identified by colorimetric screening on X gal containing indicator plates The isolated plasmid DNA was characterised by agarose gel electrophoresis and restriction enzyme analysis Analysis of the isolated plasmid DNA by agarose gel electrophoresis indicated that in contrast to parental non recombinant vector DNA that yielded three bands the putative recombinant plasmid DNA consistently yielded eight distinct bands In addition digestion of the putative recombinant plasmid DNA with *Eco*RI which flanks the insertion site yielded the same banding pattern as for the control uncut DNA In contrast digestion of the parental plasmid DNA yielded a single DNA fragment of approximately 3 kb which corresponded with the expected size of the linearised parental plasmid (results not shown) The results suggested that the presence of the *rhlAB* fragment may in some way destabilise the vector Thus to determine whether the plasmid DNA isolated from the *lac* transformants was indeed recombinant the parental and recombinant plasmid DNAs were digested with *Sca*I which cuts once in the vector borne ampicillin resistance gene In contrast to the parental plasmid DNA which yielded an expected 3 kb band digestion of the recombinant plasmid DNA yielded a single band of 2 6 kb indicating that the plasmid did not contain the *rhlAB* DNA fragment and in fact that 400 bp of the vector sequence was removed The nature of the instability of the pGEM® T Easy vector remains a subject of speculation but it may be that the high G+C content of the *rhlAB* DNA fragment (67%) compared to the G+C content of *E coli* genomic DNA (51%) interfered with optimal plasmid replication

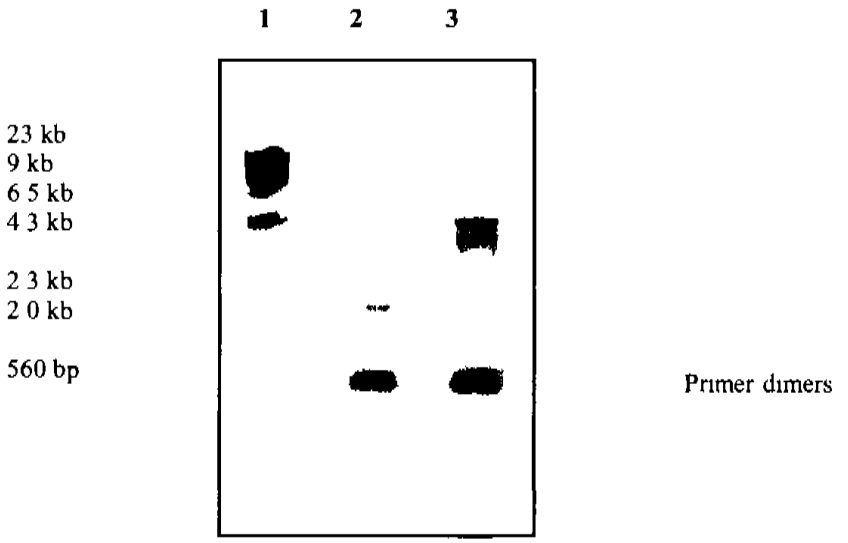


Fig 3 4 Agarose gel electrophoretic analysis of the amplicons obtained by PCR amplification of the *P aeruginosa* PAO1 *rhlAB* genes using primers FFP1 and MRP Lane 1 DNA molecular marker lane 2 amplicon obtained using SUPERTHERM DNA polymerase lane 3 amplicon obtained using SUPERTHERM DNA polymerase and the addition of 5 / (v/v) DMSO The sizes of the DNA molecular weight marker phage λ digested with *Hind*III are indicated to the left of the figure

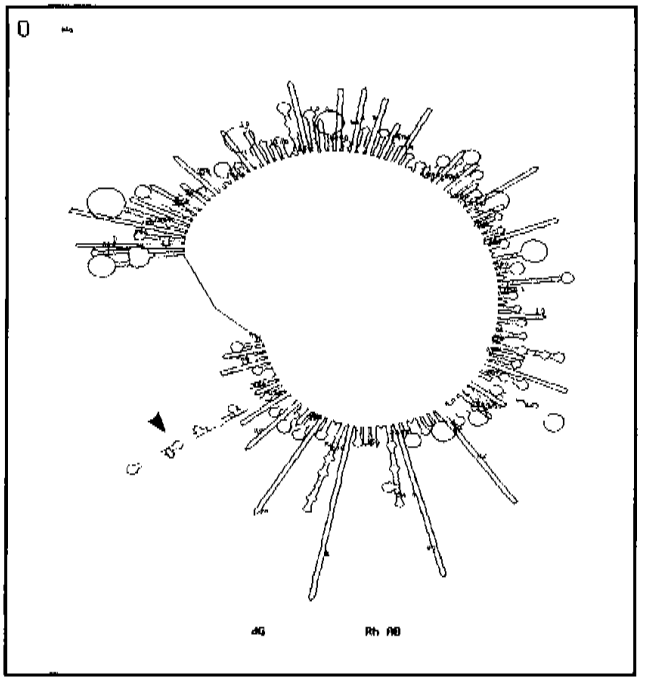


Fig 3 5 Secondary structure of the 2 3 kb *rhlAB* amplicon at 72 C using MFOLD (available online at http //mfold2 wustl edu/~mfold/dna/form1 cgi) The arrow indicates the large hairpin loop structure at nucleotides 400 - 500 which might have been responsible for detachment of the DNA polymerase from the template during the elongation step thereby resulting in an amplicon of 500 bp

Based on the above results a strategy was adopted whereby the *rhlAB* DNA fragment was to be cloned into the cosmid vector pLAH (Bertani *et al* 1999) It was reasoned that the larger cosmid vector (22 kb) would be able to more stably maintain the cloned insert DNA The *rhlAB* genes were therefore amplified from the *P aeruginosa* PAO1 genomic DNA using primers ForBgl and RevHind These primers were identical in sequence to those previously used except that they were extended at their 5 ends by additional nucleotides which incorporated unique restriction enzyme recognition sites to facilitate subsequent cloning procedures (Table 3 1)

By making use of genomic DNA isolated from *P aeruginosa* PAO1 and oligonucleotide primers ForBgl and RevHind PCR amplification was performed using the optimised conditions as described under Materials and Methods (Section 3 2 3 2) The amplicon was subsequently digested with both *Bgl*II and *Hind*III and cloned into pLAH31 which had been digested with both *Bam*HI and *Hind*III Although *Bgl*II and *Bam*HI generate compatible 5 ends the resultant hybrid site does not constitute a target for either of the restriction enzymes Thus recombinant transformants resulting from the transformation of competent *E coli* DH5α cells were identified by PCR analysis of the extracted plasmid DNA using primers ForBgl and RevHind A plasmid from which a 2 3 kb DNA fragment was amplified was selected and designated pLAH AB The terminal 400 – 500 nucleotides at both ends of the cloned insert DNA were characterised by automated DNA sequencing procedures using ForBgl and RevHind as sequencing primers Comparison of the sequences obtained to the *P aeruginosa* genome sequence (www pseudomomas com) by BLASTN homology searches indicated that the sequences displayed identity to the corresponding terminal sequences of the targeted *rhlAB* genes located in the *P aeruginosa* genome

3 3 1 1 2 Construction of the allelic exchange vector pLAH ABK

Plasmids pLAH AB and pUC4K harbouring a kanamycin resistance cassette (Vieira and Messing 1982) served as sources for the construction of the allelic exchange vector pLAH ABK Digestion of recombinant plasmid pLAH AB with *Pst*I which cuts once in each the *rhlA* and *rhlB* genes yielded two DNA fragments of approximately 23 and 1 1 kb respectively The larger of the two DNA fragments was purified from the agarose gel and used in subsequent ligation reactions The 1 2 kb kanamycin cassette was recovered from pUC4K by digestion with *Pst*I gel purified and ligated into the deletion site of the pLAH AB DNA (Fig 3 1) Following transformation of competent *E coli* DH5α cells with the ligation reaction mixture the plasmid DNA was extracted from kanamycin resistant transformants and characterised by PCR analysis followed by restriction

enzyme analysis of the amplicons (Fig 3 6) PCR analysis using primers ForBgl and RevHind and either parental or recombinant plasmid DNA as template yielded similarly sized amplicons Thus to verify the successful cloning of the kanamycin resistance cassette the amplicons were subsequently digested with *Pst*I By contrast to the digested *rhlAB* amplicon which yielded restriction fragments of approximately 0 45 0 72 and 1 1 kb (Fig 3 6 lane 9) digestion of the *rhlAB kan*^{R} amplicons yielded restriction fragments of approximately 0 45 0 72 and 1 2 kb (Fig 3 6 lanes 3 5 and 7) The latter fragments correspond with the size of the kanamycin resistance cassette (1 2 kb) together with 720 bp of upstream and 450 bp of downstream *P aeruginosa* DNA flanking the insertion A recombinant plasmid was designated pLAH ABK and selected for further use

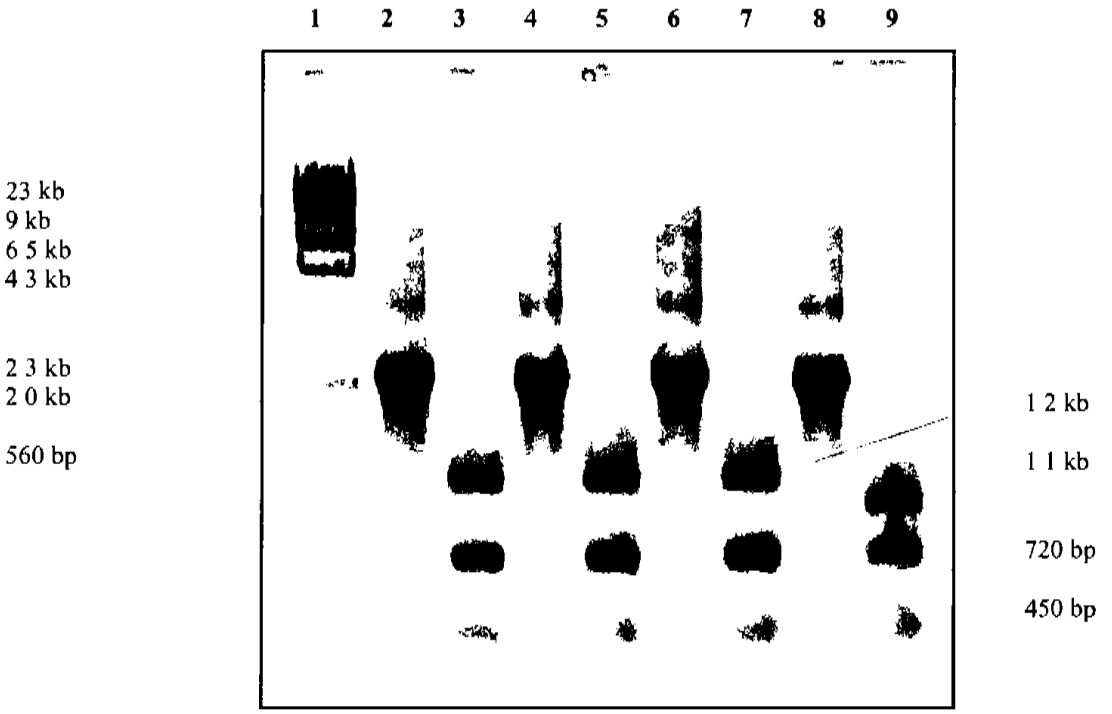


Fig 3 6 Agarose gel electrophoretic analysis of the amplicons obtained by PCR amplification of the cloned *rhlAB kan*^{R} cassette in pLAH ABK and restriction analysis of the amplicons Lane 1 DNA molecular marker Lanes 2 4 and 6 amplified insert from putative recombinant pLAH ABK plasmid constructs Lanes 3 5 and 7 restriction of the amplicons with *Pst*I The amplicon resulting from PCR amplification of the cloned *rhlAB* genes in pLAH AB (lane 8) followed by *Pst*I digestion of the amplicon (lane 9) are also shown The sizes of the DNA molecular weight marker phage λ digested with *Hind*III are indicated to the left of the figure while the sizes of the restriction DNA fragments are indicated to the right of the figure

3 3 1 1 3 Generation of isogenic mutant strains of P aeruginosa PAO1

To generate *P aeruginosa* strains harbouring the *rhlAB kan*^{R} null allele the recombinant broad host range allelic exchange vector pLAH ABK (IncP) was introduced into wild type *P aeruginosa* PAO1 by triparental mating and recipient strains were selected based on resistance to neomycin and tetracycline Plasmid pPH1JI (IncP) which belongs to the same incompatibility group as pLAH31 and harbours a gentamicin marker was then conjugated into the recipient *P aeruginosa* PAO1 strain carrying the pLAH ABK plasmid by triparental mating Exconjugants that may have integrated the *rhlAB kan*^{R} allele into the *P aeruginosa* chromosome but have lost the pLAH plasmid backbone were selected by plating on LB agar plates supplemented with neomycin gentamicin and ampicillin (to counterselect against donor *E coli* cells) Single colonies were subsequently streaked on LB agar plates supplemented with tetracycline to verify loss of the pLAH plasmid backbone However of the more than 100 colonies screened none were sensitive to tetracycline These results suggested that the pLAH ABK plasmid may have been integrated into the *P aeruginosa* genome by homologous recombination via a single crossover event Such a conclusion is however subject to verification by Southern blot analysis Due to the apparent lack of double reciprocal mutant strains this strategy was not pursued further

3 3 2 Generation of *rhlAB* deficient *P aeruginosa* PAO1 strains based on the use of an allelic exchange vector containing a counter selectable marker

An allelic exchange strategy based on selecting for the loss of integrated plasmid DNA backbone from the bacterial chromosome following insertional inactivation of the chromosomal *rhlAB* genes was next investigated For this purpose the counter selectable marker gene *sacB* was incorporated onto the allelic exchange plasmid In some studies this strategy has been reported to achieve a 100% efficiency in generating double crossover mutants (Schweizer 1992 Schweizer and Hoang 1995) Results obtained previously (Section 3 3 1 1 1) indicated that cloning of the *rhlAB* DNA fragment into ColE1 based vectors (e g pUC pGEM® T Easy) led to instability of the recombinant plasmid thus excluding their use as suicide vectors for allelic replacement In addition as a consequence of its size plasmid pLAH ABK (25 kb) was not amenable to easy genetic manipulation Therefore plasmid pPT212 was used to construct a second allelic exchange vector The plasmid possesses several properties that makes it suitable as a cloning vector These include a vegetative origin of replication (*oriV*) that permits replication in various gram negative bacteria an *oriT* that permits transfer of the plasmid DNA via conjugation a gentamicin antibiotic marker gene

and the multiple cloning site (MCS) from pUC118 which increases the number of potential cloning strategies available by extending the range of enzymes that can be used to generate restriction fragments suitable for cloning (Totten *et al* 1990)

3 3 2 1 Construction of pPTSac

To construct plasmid pPTSac which would be used as cloning vector for genes targeted for allelic exchange plasmid pFLP2 served as the source for the counter selectable *sacB* gene and plasmid pPT212 served as the backbone of the new vector construct Towards constructing plasmid pPTSac recombinant plasmid pPT212 which harbours a cloned *rpoN* cassette was first digested with *Bam*HI to excise the *rpoN* cassette after which the purified vector DNA was self ligated to yield plasmid pPT212S To complete the construction of pPTSac the *sacB* gene was recovered from plasmid pFLP2 as a 2 6 kb *Xba*I – *Hind*III DNA fragment and ligated into similarly prepared pPT212S vector DNA After transformation of competent *E coli* DH5α cells recombinant plasmid DNA was extracted from gentamicin resistant transformants and characterised by restriction enzyme digestion Digestion of the plasmid DNA with both *Xba*I and *Hind*III resulted in DNA fragments corresponding to the size of the pPT212S vector DNA (8 kb) and a *sacB* containing DNA fragment (2 6 kb) (Fig 3 7 lane 4)

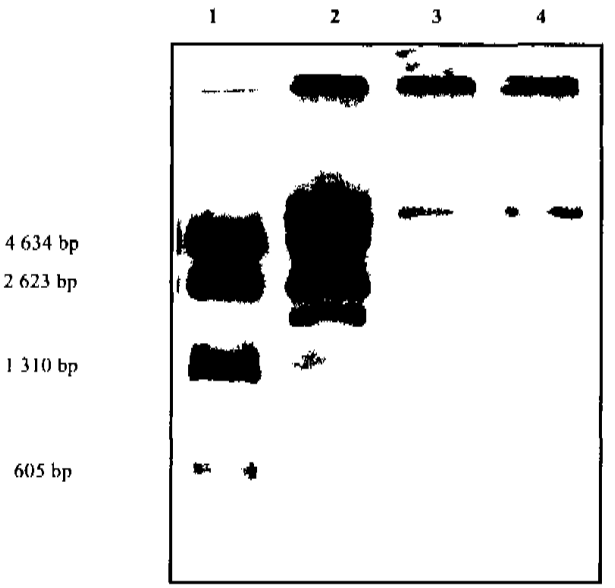


Fig 3 7 Agarose gel electrophoretic analysis of recombinant plasmid pPTSac Lane 1 DNA molecular marker lane 2 pFLP2 digested with both *Xba*I and *Hind*III lane 3 pPT212S vector DNA digested with *Xba*I and *Hind*III lane 4 recombinant plasmid pPTSac digested with both *Xba*I and *Hind*III The sizes of the DNA molecular marker plasmid pFLP2 digested with both *Bam*HI and *Hind*III are indicated to the left of the figure

3 3 2 2 Construction of allelic exchange vector pPTS-ABK

Due to the cloning strategy used to construct the pLAH ABK allelic exchange vector the 2 4 kb *rhlAB kan*^{R} cassette could not be excised from the vector In addition the MCS of plasmid pPTSac lacked a *Hind*III recognition site and necessitated that the *rhlAB kan*^{R} cassette be cloned into the *Sma*I site of pPTSac as a blunt ended DNA fragment (Fig 3 2) The *rhlAB kan*^{R} cassette was subsequently obtained by PCR amplification using pLAH ABK as template DNA together with primers ForBgl and RevHind *Pwo* DNA polymerase was used for this purpose as it possesses a 3 to 5 exonuclease activity and therefore results in blunt ended PCR amplicons The obtained 2 4 kb amplicon was purified from an agarose gel and then cloned into *Sma*I digested pPTSac to yield plasmid pPTS ABK Since the cloning procedure did not result in the restoration of the *Sma*I recognition sequence the plasmid DNA from neomycin resistant transformants were extracted and characterised by PCR analysis By making use of primers ForBgl and RevHind an amplicon of 2 4 kb was obtained when recombinant pPTS ABK plasmid DNA was used as template in the PCR reaction (Fig 3 8 lane 3) No amplification occurred in the control PCR reaction in which pPTSac was used as template (Fig 3 8 lane 2) These results therefore served to confirm the successful cloning of the *rhlAB kan*^{R} cassette into plasmid pPTSac

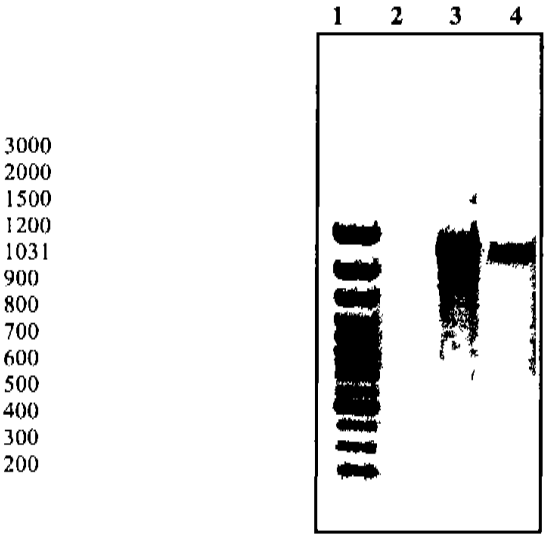


Fig 3 8 Agarose gel electrophoretic analysis indicating amplification of the cloned *rhlAB kan*^{R} cassette in the recombinant pPTS ABK plasmid construct using primers ForBgl and RevHind Lane 1 DNA molecular marker lane 2 control reaction containing pPTSac as template DNA lane 3 amplified insert from pPTS ABK lane 4 amplicon using *P aeruginosa* PAO1 genomic DNA as template The sizes (in base pairs) of a GeneRuler^{TM} 100 bp ladder (Fermentas) are indicated to the left of the figure

3 3 2 3 Generation of isogenic mutant strains of P aeruginosa PAO1

To generate *P aeruginosa* PAO1 strains harbouring the *rhlAB kan*^{R} null allele by homologous recombination via a double crossover event the recombinant pPTS ABK allelic exchange vector was introduced into wild type *P aeruginosa* PAO1 by triparental mating and strains harbouring an integrated copy of the mutant allele were selected in a single step by plating on LB agar plates supplemented with neomycin and 5% (w/v) sucrose Following incubation overnight single colonies were selected and replica plated on LB agar plates supplemented with gentamicin Of the 12 sucrose and kanamycin resistant colonies screened four (25%) displayed sensitivity to gentamicin These results suggested that the *rhlAB kan*^{R} null allele was integrated into the genome of these four strains via a double crossover event

3 3 2 4 Southern blot analysis of putative rhlAB mutants of P aeruginosa generated by the use of a counter-selectable marker

To determine whether integration of the mutant *rhlAB kan*^{R} allele occurred by means of a single or double crossover event Southern blot analysis was performed The genomic DNA of the four mutant *P aeruginosa* PAO1 strains identified above was extracted digested with *Pst*I and then subjected to Southern blot hybridisation using a DIG dUTP labelled *rhlAB* amplicon as probe In this analysis *Pst*I digested genomic DNA of *P aeruginosa* PAO1 was included as a positive hybridisation control and that of *E coli* DH5α as a negative hybridisation control Digestion of wild type *P aeruginosa* PAO1 genomic DNA with *Pst*I should yield in addition to numerous other restriction fragments restriction fragments of 5 5 1 1 and 2 kb that span the integration region on the chromosomal DNA However digestion of the genomic DNA of mutant strains in which the *rhlAB kan*^{R} allele was integrated into the genome via a double crossover event would yield similar restriction DNA fragments for the corresponding genomic region except that the 1 1 kb *rhlAB* specific DNA fragment would be replaced with a 1 2 kb kanamycin cassette that had been used to inactivate the *rhlAB* genes Since the *rhlAB* specific probe would not be expected to hybridise to the 1 2 kb band it served to distinguish mutant strains from the wild type strain

The results indicated that the probe did not hybridise to the *Pst*I digested chromosomal DNA of *E coli* DH5α (Fig 3 9 lane 6) However the *rhlAB* probe hybridised with DNA restriction fragments of 5 5 1 1 and 2 kb from both the wild type PAO1 strain (Fig 3 9 lane 1) and putative mutant strains (Fig 3 9 lanes 2 5) These results suggested that mutant strains were either not generated or that mutant strains were generated via a single crossover event However in both instances the

results are contradictory to the phenotypic characteristics of the mutant strains which indicated that they had lost the plasmid backbone (sucrose^R and Gm^S) but maintained the integrated mutant allele (Kan^R)

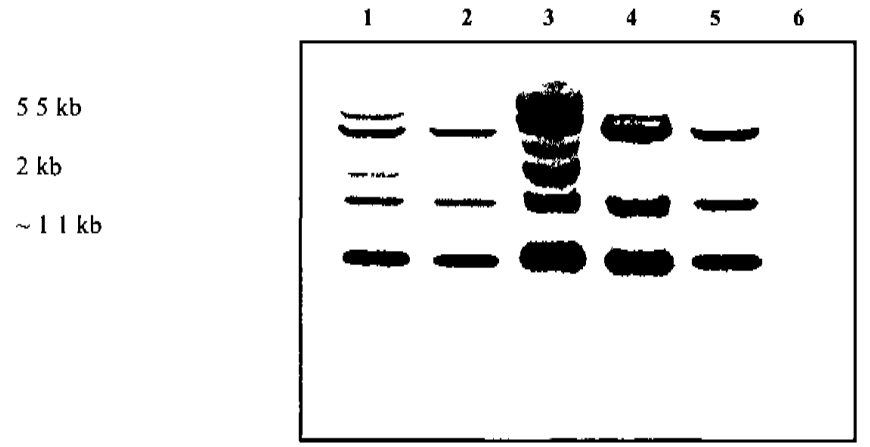


Fig 3 9 Southern blot analysis of genomic DNA extracted from putative *P aeruginosa* mutant strains Genomic DNA extracted from the wild type *P aeruginosa* PAO1 (lane 1) and mutant strains D1 (lane 2) D2 (lane 3) D3 (lane 4) and D4 (lane 5) were digested with *Pst*I resolved by agarose gel electrophoresis and transferred onto a nylon membrane *Pst*I digested *E coli* genomic DNA (lane 6) was included as a negative hybridisation control The membrane was probed with a DIG labelled *rhlAB* amplicon

BLASTN analysis indicated that *rhlAB* which was used as probe does not display significant similarity to the 1 2 kb kanamycin cassette and it therefore appears unlikely that the hybrised band in the mutant strains might be due to the probe hybridising to the kanamycin cassette More likely digestion of the genomic DNA with *Pst*I might have generated several DNA fragments of 1 1 1 2 kb that co migrated with the expected DNA bands One of these genomic DNA bands might have hybridised with the *rhlAB* probe thus yielding a positive signal and thereby masking the absence of the 1 2 kb band in the mutant strains Due to the ambiguity of the results and low efficiency this approach was not pursued further

3 3 3 Generation of *rhlAB* deficient *P aeruginosa* PAO1 strains based on plasmid incompatibility and the use of a counter selectable marker

To increase the efficiency of generating a double crossover mutant a new strategy was developed by combining the two approaches previously used i e plasmid incompatibility and the use of *sacB* as counter selectable marker (Fig 3 10) For this purpose recombinant allelic exchange vector pPTS

ABK was used (Section 3.3.2.2) and an incompatible "chase" plasmid (pJBSac), containing a cloned copy of the *sacB* counter-selectable marker gene, was constructed as described below. By including the *sacB* counter-selectable marker gene on both the allelic exchange vector and "chase" plasmid, it would be possible to remove both plasmids from the bacterial cell in a single step.

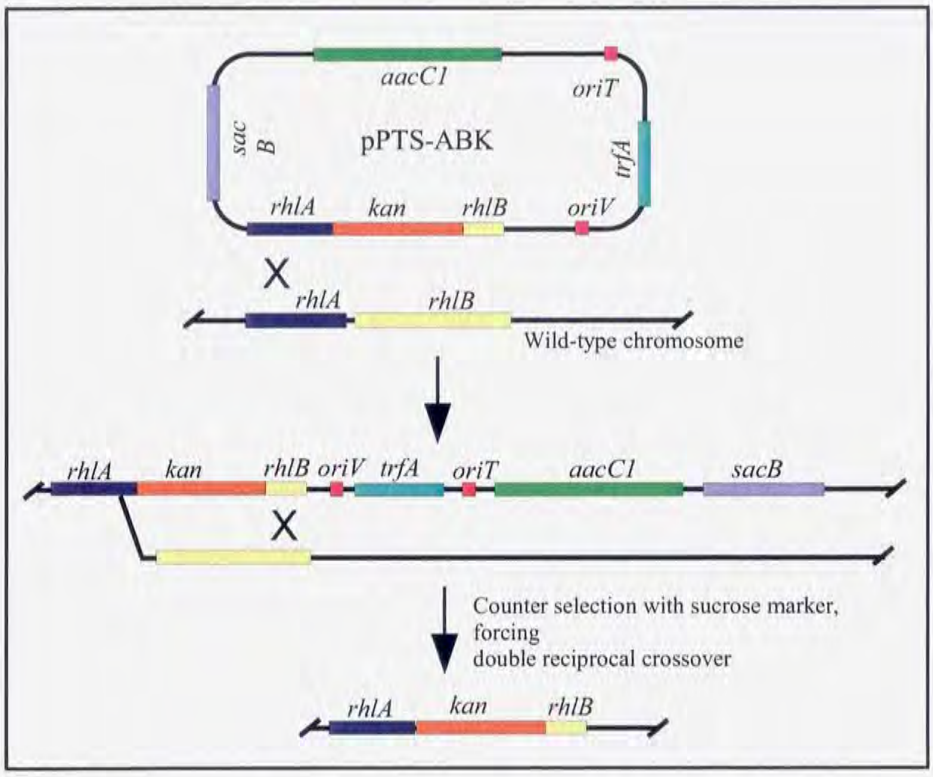


Fig. 3.10 Schematic representation of the theoretical basis for creating a double reciprocal mutant. The first step illustrates the integration of the conjugally transferred pPTS-ABK into the homologous chromosomal region of the wild-type strain PAO1. The resulting merodiploid strain is Gm^R, Kan^R and $sucrose^S$. The second step depicts the excision of unwanted DNA sequences by promoting sucrose resistance. The resulting strain is only Kan^R and the *rhlAB* operon is inactivated.

3.3.3.1 Construction of plasmid pJBSac

Plasmid pFLP2, which harbours a cloned copy of the *B. subtilis sacB* gene, and the broad-host-range plasmid pJB3Tc20 were used as sources for the construction of the chase plasmid pJBSac. Plasmid pJB3Tc20 was chosen for this purpose, as it contains a vegetative origin of replication (*oriV*) that permits replication in different gram-negative bacteria, including *E. coli* and *P. aeruginosa*, an *oriT* that permits introduction of the plasmid DNA into *P. aeruginosa* by triparental mating, as well as ampicillin and tetracycline antibiotic marker genes (Blatny *et al.*, 1997).

To construct pJBSac (Fig 3 3) plasmid pFLP2 was digested with *Eco*RV and the excised 3 kb DNA fragment containing the *sacB* gene was purified from the agarose gel and cloned into *Eco*RV digested pJB3Tc20 vector DNA As the *Eco*RV site is situated in the *tetA* gene of plasmid pJB3Tc20 recombinant transformants resulting from transformation of competent *E coli* DH5α cells were readily identified by replica plating on LB agar plates supplemented with either ampicillin or tetracycline Plasmid DNA was subsequently extracted from transformants displaying an Ap^R/Tc^S phenotype and digestion of the plasmid DNA with *Eco*RV resulted in the excision of a 3 kb DNA fragment indicating that the *sacB* containing DNA fragment had been cloned successfully (Fig 3 11 lane 4)

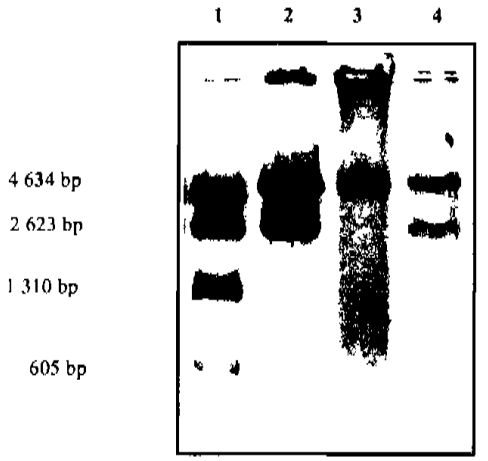


Fig 3 11 Agarose gel electrophoretic analysis of recombinant plasmid pJBSac Lane 1 DNA molecular marker lane 2 pFLP2 digested with *Eco*RV lane 3 pJB3Tc20 vector DNA linearised by digestion with *Eco*RV lane 4 recombinant plasmid pJBSac digested with *Eco*RV The sizes of the DNA molecular marker plasmid pFLP2 digested with both *Bam*HI and *Hind*III are indicated to the left of the figure

3 3 3 2 Generation of isogenic mutant strains of P aeruginosa PAO1

The allelic exchange plasmid pPTS ABK (IncP) was introduced into wild type *P aeruginosa* PAO1 by triparental mating and selected for by plating on LB agar plates supplemented with gentamicin and neomycin Plasmid pJBSac (IncP) was then introduced into the recipient *P aeruginosa* strain containing pPTS ABK and the presence of both plasmids was selected for by plating on LB agar plates supplemented with carbenicillin and neomycin Following incubation overnight at 37 C single colonies were streaked on Pseudomonas Isolation Agar (Difco) containing 5% sucrose (w/v) and neomycin The plates were incubated at 37 C until colonies became visible and several of these were restreaked on the same selective medium The sucrose resistant colonies were subsequently plated in duplicate on LB agar plates containing gentamicin (to counter select pJBSac) neomycin

(to select for the integration cassette) or carbenicillin (to counter select pPTSac ABK backbone) Of the 48 sucrose resistant colonies screened 43 (90%) were resistant to neomycin but sensitive to both gentamicin and carbenicillin These results indicated that both the chase plasmid pJBSac and the backbone of the allelic exchange vector pPTS ABK were lost from the strains and that the *rhlAB kan*^{R} null allele was integrated into the *P aeruginosa* genome via a double crossover event

3 3 3 3 Southern blot analysis of putative P aeruginosa rhlAB mutants generated through the use of incompatible plasmids and a counter-selectable marker

To determine whether integration of the mutant null allele occurred by means of a single or double crossover event Southern blot analysis was performed The chromosomal DNA of 12 randomly selected mutant strains was isolated digested with *Pst*I and separated by agarose gel electrophoresis The DNA fragments were transferred onto a nylon membrane by capillary blotting and the membrane was then hybridised with a DIG dUTP labelled DNA probe specific for *rhlAB* The results indicated that the *rhlAB* specific probe (Fig 3 12a) hybridised with *Pst*I digested chromosomal DNA of the wild type *P aeruginosa* PAO1 strain as well as with DNA restriction fragments from each of the putative mutant strains These results were identical to those presented earlier (Fig 3 9) suggesting that mutant strains were either not generated or that mutant strains were generated via a single crossover event As expected the probe did not hybridise to *Sac*I linearised pJB3Tc20 and pPTSac plasmid DNA (Fig 3 12a lanes 12 and 13 respectively)

Since the results obtained by Southern blot analysis appeared to be contradictory to the phenotypic characteristics of the mutant strains Southern blot hybridisation was subsequently performed using pPTSac plasmid DNA as labelled probe to determine whether integration occurred by means of a single crossover event One of the mutant strains (K6) was selected and the genomic DNA was isolated and then digested with *Ava*I In this analysis *Ava*I digested chromosomal DNA of the wild type *P aeruginosa* PAO1 strain was included as a negative hybridisation control while pPTSac plasmid DNA was included as a positive hybridization control The probe specific for the pPTSac vector DNA hybridised to pPTSac plasmid DNA only (Fig 3 12b lanes 1 and 4) The labelled probe did not hybridise with the digested chromosomal DNA of either the wild type PAO1 or mutant K6 strains (Fig 3 12b lanes 2 and 3 respectively) These results therefore suggested that the mutant *rhlAB kan*^{R} allele was integrated into the chromosomal DNA of the mutant K6 strain by means of a double crossover event

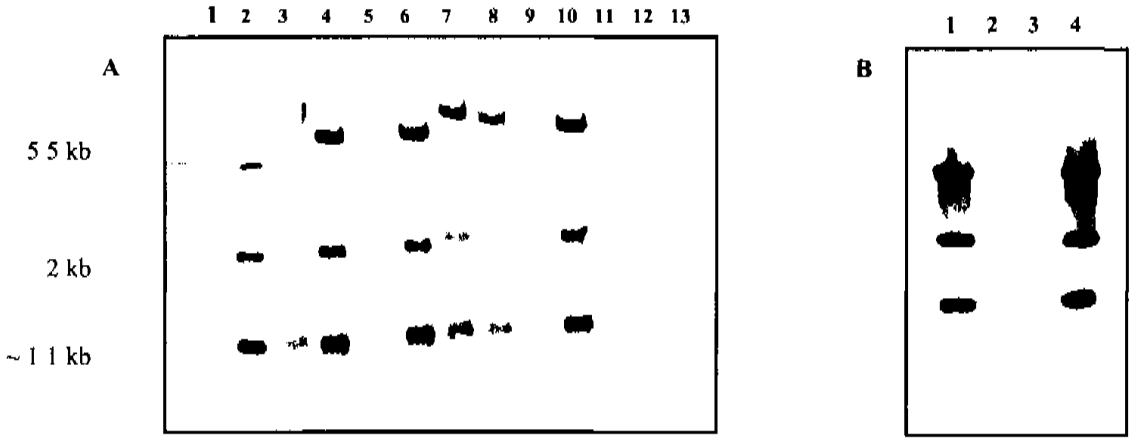


Fig 3 12a Southern blot analysis of genomic DNA of putative double reciprocal mutants (K1 – K11) using a DIG labelled *rhlAB* amplicon as probe Genomic DNA extracted from the wild type *P aeruginosa* (lane 11) and the mutant strains K1 K2 K3 K4 K5 K6 K8 K9 K10 and K11 (lanes 1 to 10) were digested with *Pst*I resolved by agarose gel electrophoresis and transferred onto a nylon membrane Plasmids pJB3Tc20 (lane 12) and pPTSac (lane 13) were linearised by digestion with *Sac*I **Fig 3 12b** Southern blot analysis of genomic DNA extracted from the *P aeruginosa* mutant strain K6 Genomic DNA extracted from the wild type *P aeruginosa* PAO1 (lane 2) and mutant strain K6 (lane 3) were digested with *Ava*I resolved by agarose gel electrophoresis and transferred onto a nylon membrane Undigested plasmid pPTSac (lanes 1 and 4) was included as a positive control The membrane was probed with DIG labelled pPTSac vector DNA

3 3 3 4 Rhamnolipid assay on merodiploids

To determine whether the chromosomal *rhlAB* genes had been replaced with the *rhlAB kan*^{R} null allele a few of the mutant strains generated in the previous section were selected and subjected to a rhamnolipid assay For rhamnolipid assays the wild type *P aeruginosa* PAO1 mutant *P aeruginosa* strain SS1258 (Table 3 1) and putative mutant strains constructed in the preceding section (K1 through K6) were inoculated into PPGAS medium lacking antibiotics and incubated at 37 C for 72 h *P aeruginosa* PAO1 was included in these assays as a control to determine the wild type level of rhamnolipid synthesis whereas the *rhlAB* deficient SS1258 strain served as a negative control for rhamnolipid synthesis The rhamnolipid assays were performed in triplicate as described under Materials and Methods (Section 3 2 15) and the results are presented in Fig 3 13

With the exception of mutant strain K3 none of the other mutant strains synthesised detectable levels of rhamnolipid Since *rhlA* has been reported to be essential for rhamnolipid synthesis (Ochsner *et al* 1994 Rahim *et al* 2001) these results indicated that the mutant *rhlAB kan*^{R} allele was integrated into the genomic DNA of the respective strains Although Southern blot analysis

using *rhlAB* as labelled probe (Fig. 3.12a), did not allow clear distinction as to whether integration of the mutant null allele occurred by means of a single or double crossover event, two lines of evidence support integration of the mutant allele into the genome of these strains by means of a double crossover event. This is based on the observations that all of these strains were sensitive to gentamicin, but displayed resistance to neomycin (Section 3.3.3.2); and Southern blot analysis, using plasmid pPTSac as labelled probe, did not hybridise to the digested genomic DNA of strain K6 (Fig. 3.12b), thus indicating that the plasmid DNA backbone, containing the Gm^R marker, was lost from the strain.

Interestingly, mutant strain K3 produced rhamnolipid and the level was similar to that synthesised by the wild-type *P. aeruginosa* PAO1 strain. This may suggest that integration of the mutant *rhlAB*::*kan*^{R} allele may have occurred at a different position in the genome, thus leaving an intact copy of the *rhlAB* genes on the bacterial genome, and thus resulting in synthesis of rhamnolipid. Alternatively, strain K3 may have been contaminated with wild-type PAO1, as the mutant strains were cultured in the absence of antibiotics.

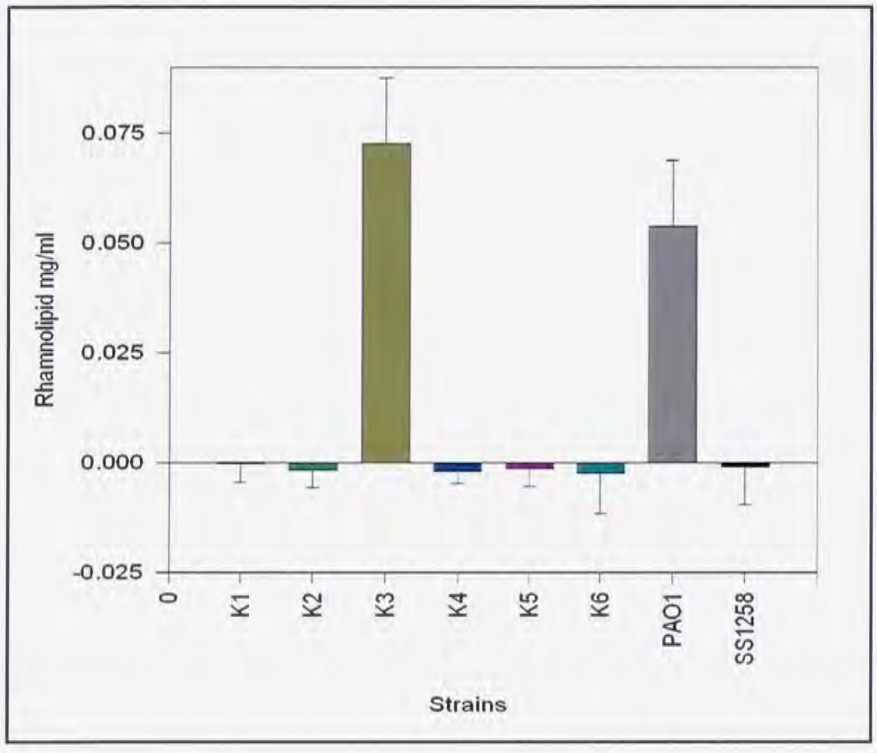


Fig. 3.13 Graph displaying rhamnolipid synthesis of different mutant *P. aeruginosa* strains (K1 to K6). *P. aeruginosa* was used as a positive control, whereas *P. aeruginosa* SS1258, a *rhlAB*-deficient strain, was used as a negative control. Error bars denote one standard deviation of the mean.

3 4 CONCLUSIONS

Towards investigating the role of rhamnolipid in maintaining the *P aeruginosa* biofilm architecture different strategies were used in an effort to generate mutant *P aeruginosa* PAO1 strains containing insertionally inactivated *rhlAB* genes Whereas a strategy based on the use of incompatible plasmids did not yield double reciprocal mutants a second strategy based on the use of the *Bacillus subtilis sacB* gene as a counter selectable marker resulted in 25% of the derived strains being double reciprocal mutants The yield of double reciprocal mutants was however improved by using incompatible plasmids that both contained the *sacB* counter selectable marker gene This approach resulted in a significantly higher number of double reciprocal mutants (90%) Five of the six mutant strains generated by the latter approach were furthermore shown to be deficient in rhamnolipid synthesis thus confirming successful inactivation of the chromosomal borne *rhlAB* genes Despite the phenotypic properties of the resultant mutant strains having indicated that the strains were generated via double crossover events Southern blot analysis using the *rhlAB* amplicon as probe together with *Pst*I digested wild type *P aeruginosa* PAO1 and mutant genomic DNA yielded ambiguous results This made it difficult to confirm whether the mutant strains indeed resulted from a double crossover event To obtain unambiguous results Southern blot analysis should in future be performed by using the kanamycin resistance cassette and/or the 1 1 kb *rhlAB* specific insert which had been excised during construction of the *rhlAB kan*^{R} null allele as probes These would verify the presence of the kanamycin cassette in the genomic DNA of mutant strains whilst lack of hybridisation with the 1 1 kb *rhlAB* probe would indicate integration of the *rhlAB kan*^{R} null allele into the intended target site A probe comprising the genetic backbone of the allelic exchange vector can also be used to verify its presence or absence from the genomic DNA and thus indicate whether the strains were generated via a single or double crossover event Alternatively PCR may also be used to amplify the region spanning the integration site

The *rhlAB* mutant strains generated in this part of the study were to be used in investigations regarding the role of rhamnolipid in *P aeruginosa* PAO1 biofilm development and maintenance of its biofilm architecture However after having constructed the *rhlAB* mutant strains and just prior to undertaking these studies Davey *et al* (2003) published a paper detailing the influence of rhamnolipid on *P aeruginosa* PAO1 biofilm architecture Consequently the mutant strains were not used in this study and the aims of the study were altered to rather focus on the transcriptional regulation of the *rhlAB* promoter during biofilm development as detailed in the following Chapter

CHAPTER 4

TRANSCRIPTIONAL ACTIVITY AND REGULATION OF THE *Pseudomonas aeruginosa* PAO1 *rhlAB* PROMOTER DURING BIOFILM FORMATION

4 1 INTRODUCTION

In the vast majority of ecological niches *P aeruginosa* can grow in association with surfaces (Costerton *et al* 1995) Such surface associated growth leads to the formation of biofilms that are defined as organised communities of cells embedded in an extracellular matrix attached to a biotic or abiotic surface (Costerton *et al* 1994 Molin and Tolker Nielsen 2003) In *P aeruginosa algC* (Davies and Geesey 1992) and *algD* (Hoyle *et al* 1993) were the first genes reported to be up regulated in biofilm growing cells Numerous other genes (O Toole and Kolter 1998 Whiteley *et al* 2001) and proteins (Sauer *et al* 2002) have since been identified that are involved in the initial attachment of bacteria to surfaces and the subsequent formation of well developed biofilms Recent studies have also linked quorum sensing and biofilm formation (Davies *et al* 1998)

P aeruginosa possesses two quorum sensing systems namely *las* and *rhl* These systems form part of a global regulatory network and control the expression of numerous genes (Whiteley *et al* 1999 Schuster *et al* 2003 Wagner *et al* 2003) The two quorum sensing systems are inter related in that LasR activates the expression of *rhlR* and *rlhI* (Pesci *et al* 1997) Each quorum sensing system consists of a transcriptional activator LasR (Gambello *et al* 1993) or RhlR (Brint and Ohman 1995) and an autoinducer synthetase LasI or RhlI Whereas LasI catalyses the synthesis of *N* (3 oxododecanoyl) L homoserine lactone ($3OC_{12}$ HSL) (Pearson *et al* 1994) RhlI catalyses the synthesis of *N* butyryl L homoserine lactone (C_4 HSL) (Pearson *et al* 1995) The transcription factors RhlR and LasR bind to a specific palindromic sequence in the promoter regions of quorum sensing controlled (*qsc*) genes known as the *lux* like box A minimal consensus sequence for the *lux* like box of *P aeruginosa* qsc promoters was defined as NNCT N_{12} AGNN (Whiteley and Greenberg 2001)

Reports regarding the role of the *las* quorum sensing system in *P aeruginosa* biofilm development appear to be ambiguous Whereas Davies *et al* (1998) reported that biofilms formed by a *P aeruginosa lasI* mutant was thinner and lacked the three dimensional architecture of the wild type PAO1 biofilm Heydorn *et al* (2002) reported no differences in the biofilm structure and density between the *P aeruginosa lasI* mutant and wild type PAO1 strains Nevertheless both *rhlI* and *lasI* may play a role in the initial steps of biofilm formation *P aeruginosa lasI* and *rhlI* mutants have been reported to be impaired in their ability to attach to a glass substratum when cultured in a glucose based medium (de Kievit *et al* 2001) Since both *P aeruginosa* quorum sensing systems

are involved in the regulation of type IV pili-mediated twitching motility (Glessner *et al.*, 1999), it may be that expression of *pilA*, which encodes the type IV pilin subunit, was down-regulated.

Recently, the role of rhamnolipid in the biofilm microcosm of *P. aeruginosa* has been investigated (Davey *et al.*, 2003). It was reported that although rhamnolipid, of which the synthesis is under the control of RhlR, was not involved in the initial attachment of *P. aeruginosa* to an abiotic surface, it is, however, responsible for the maintenance of the channels between the microcolonies. It has subsequently been proposed that rhamnolipid may prevent invading bacteria from colonising open spaces in the biofilm and it may play a role in maintaining the nutritional balance of the biofilm (Espinosa-Urgel, 2003). The promoter region of the *rhlAB* genes contains a consensus σ^{54} (RpoN) promoter sequence, which is overlapped by a consensus σ^{70} promoter sequence (Pearson *et al.*, 1997) (Fig. 4.1). The promoter region also contains a *lux*-like box where the RhlR-autoinducer complex binds to activate the transcription of the *rhlAB* genes (Pesci *et al.*, 1997; Pearson *et al.*, 1997; Medina *et al.*, 2003a).

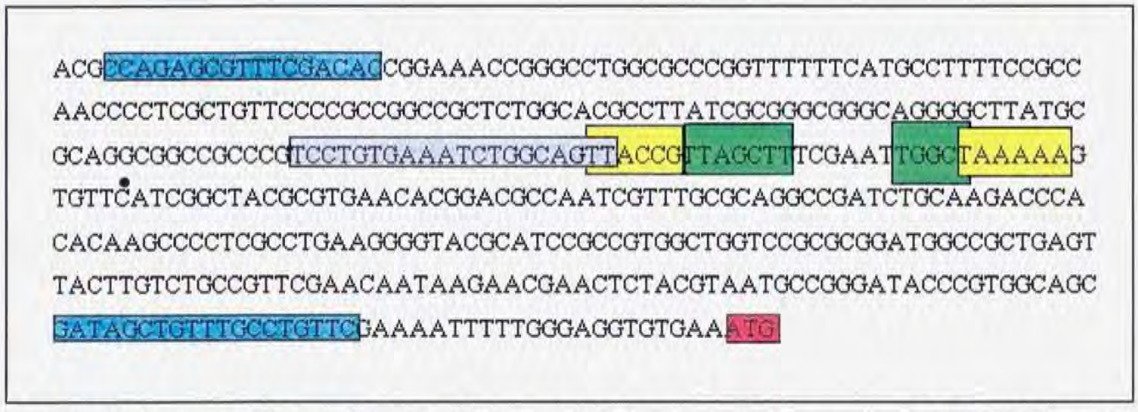


Fig. 4.1 The *rhlAB* promoter region. The annealing sites of the forward and reverse primers (AIF, AIR2) are indicated in blue (see Materials and Methods for detail), the *lux*-like box consensus sequence in purple, the σ^{70} promoter sequence in yellow and the σ^{54} promoter sequence in green. The translational start codon for RhlA is indicated in red, while the transcriptional start site (+1) is indicated by a dot. Note the overlap of the putative σ^{70} promoter sequence with both the *lux*-like box and σ^{54} promoter sequences.

Several global regulators or efflux systems have been reported to play a role in regulating quorum sensing and/or rhamnolipid synthesis. These include *nfxC* (Köhler *et al.*, 2001), Vfr (Beatson *et al.*, 2002), GacA (Parkins *et al.*, 2001), AlgQ (Ledgham *et al.*, 2003a), DksA (Jude *et al.*, 2003), QscR (Ledgham *et al.*, 2003b), MvaT (Diggle *et al.*, 2002), as well as the sigma factors RpoS (Suh *et al.*, 1999) and RpoN (Thompson *et al.*, 2003; Heurlier *et al.*, 2003). With specific reference to the

sigma factors the role of RpoN in biofilm development and especially quorum sensing has only recently been investigated Heurlier *et al* (2003) reported that RpoN exerts a global negative control on the quorum sensing machinery of *P aeruginosa* Transcriptional fusions of *rhlAB* indicated a two to four fold increase in *rhlAB* promoter activity in a *rpoN* null mutant suggesting that transcription of *rhlAB* is negatively controlled by RpoN RpoS has been implicated in regulating expression of exoproducts (Suh *et al* 1999) and quorum sensing since there is up regulation of C_4 HSL synthesis and elevated levels of RhlR RhlI regulated gene transcription (Whiteley *et al* 2000 Suh *et al* 1999) in a *rpoS* null mutant However an up regulation in the signal molecule did not result in increased levels of transcription of all genes regulated by quorum sensing implying a limited or indirect effect on gene expression

To investigate *P aeruginosa rhlAB* promoter activity during biofilm development consideration had to be given to the use of an appropriate reporter gene Several different reporter genes have been used of which the reporter protein activity can be readily quantified e g genes encoding for β galactosidase (Davies *et al* 1993) luciferase and the green fluorescent protein (Sternberg *et al* 1999 Heydorn *et al* 2000 de Kievit *et al* 2001) Of these different reporter genes the green fluorescent protein gene (*gfp*) encoding green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has become a popular reporter for the visualisation of gene expression and protein subcellular localisation (Chalfie *et al* 1994) In addition GFP does not require any other energy source or substrate except for oxygen during initial formation of the chromophore (Chalfie *et al* 1994) The aims of this part of the study were thus to investigate *P aeruginosa rhlAB* promoter activity in bacterial cells in developing and well developed biofilm structures using stable and unstable *gfp* reporter constructs For this purpose a flow cell was used to investigate gene expression in single cells and the heterogeneity of gene expression amongst various members within the biofilm population Furthermore in light of the different regulation mechanisms *rhlAB* promoter activity was also investigated in biofilms of *P aeruginosa* strains lacking either RpoN or RpoS

4 2 MATERIALS AND METHODS

4 2 1 Bacterial strains and media

The bacterial strains and plasmids used in this part of the study are listed in Table 4 1 *P aeruginosa* and *E coli* strains were maintained at 70 C as glycerol cultures and cultured in LB broth (Sambrook *et al* 1989) at 37 C with shaking at 200 rpm The following antibiotics were used to maintain the plasmid DNA and chromosomal insertions in *P aeruginosa* wild type and mutant strains tetracycline (Tc) at 100 µg/ml and gentamicin (Gm) at 50 µg/ml For plasmid DNA selection and maintenance in *E coli* the following antibiotics were used 100 µg/ml for ampicillin (Ap) and 15 µg/ml for tetracycline (Tc) For flow cell experiments and fluorometric assays the strains were cultured in ABT minimal medium (Andersen *et al* 1998) which is composed of AB minimal medium containing 0 3 mM glucose and 2 5 mg thiamine per litre medium AB minimal medium (Clark and Maaloe 1967) consists of solution A (200 ml 2 g $(NH_4)_2$ SO_4 6 g Na_2HPO_4 3 g KH_2PO_4 3 g NaCl 0 011 g Na_2SO_4) and solution B (800 ml 0 2 g $MgCl_2$ 0 01 g $CaCl_2$ 1 ml of 117 2 mg $FeCl_3$ $6H_2O$ in 250 ml [1000 x solution])

4 2 2 Genomic DNA extraction

Genomic DNA from *P aeruginosa* PAO1 was extracted according to the method of Wilson (1990) with the following modifications A single bacterial colony was inoculated into 5 ml LB broth and incubated overnight at 37 C with shaking aeration (200 rpm) The cells from 1 5 ml of the culture were collected by centrifugation at 13 000 x *g* for 2 min and suspended in 567 µl of 1 x TE buffer (10 mM Tris HCl 1 mM EDTA pH 8 0) After the addition of 30 µl of lysozyme (50 mg/ml) and 50 µl of 10% (w/v) EDTA the suspension was ineubatcd for 1 h at 4 C followed by the addition of 3 µl Proteinase K (20 mg/ml) and 30 µl of 10% (w/v) SDS Following incubation for 1 h at 37 C 100 µl of 5 M NaCl and 80 µl of a CTAB/NaCl solution (10% [w/v] CTAB in 0 7 M NaCl) were added and incubation was continued for 10 min at 65 C The chromosomal DNA was purified by extraction with an equal volume of chloroform isoamyl alcohol (24 1) followed by centrifugation for 5 min at 13 000 x *g* The supernatant was transferred to a clean microfuge tube and an equal volume of phenol chloroform isoamyl alcohol (25 24 1) was added After centrifugation as above the genomic DNA was precipitated from the recovered aqueous supernatant by the addition of 0 6 volume isopropanol The chromosomal DNA was pelleted by centrifugation for 2 min at 13 000 x *g* rinsed with 70% ethanol dried under vacuum and suspended in 80 µl 1 x TE buffer or ddH_2O An aliquot (2 µl) of the genomic DNA was analysed by electrophoresis on a 0 8% (w/v) agarose gel

Table 4 1 Bacterial strains plasmids and primers used in this study

		Relevant genotype or phenotype	**Source or reference**
Strains			
P aeruginosa	PAO1	Wild type	Holloway *et al* (1979)
	PA3B	PAO1 x DH5α(RK600) x DH5α(pC3BpRA) PAO1 with a site specific integration at *attB* with mini CTX1 containing P $_{hlAB}$ *gfp*mut3	This study
	PA113	PAO1 x DH5α(RK600) x DH5α(pC113pRA) PAO1 with a site specific integration at *attB* with mini CTX1 containing P $_{hlAB}$ *gfp*(ASV)	This study
	PAO1Δ*rpoN*	PAO1UNSW Δ*rpoN aacC1* Gm^R	Thompson *et al* (2003)
	N3B	PAO1Δ*rpoN* x DH5α(RK600) x DH5α(pC3BpRA) PAO1Δ*rpoN* with a site specific integration at *attB* with mini CTX1 containing P $_{hlAB}$ *gfp*mut3	This study
	N113	PAO1Δ*rpoN* x DH5α(RK600) x DH5α(pC113pRA) PAO1Δ*rpoN* with a site specific integration at *attB* with mini CTX1 containing P $_{hlAB}$ *gfp*(ASV)	This study
	SS24	PAO1 Δ*rpoS101 aacC1* Gm^R	Suh *et al* (1999)
	S3B	SS24 x DH5α(RK600) x DH5α(pC3BpRA) SS24 with a site specific integration at *attB* with mini CTX1 containing P $_{hlAB}$ *gfp*mut3	This study
	S113	SS24 x DH5α(RK600) x DH5α(pC113pRA) SS24 with a site specific integration at *attB* with mini CTX1 containing P $_{hlAB}$ *gfp*(ASV)	This study
E coli	DH5α	F *recA1 endA1 hsdR17 deoR thi 1 supE44 gyrA96 relA1* Δ(*lacZYA argF*) U169 λ [Φ80d*lacZ*ΔM15]	Sambrook *et al* (1989)
Plasmids			
	CTX3B	Tc^R mini CTX1 cloning vector with a *gfp*mut3 cassette cloned on a *Spe*I RBSII *gfp*mut3 T_0T_1 *Not*I fragment from pBS3B	Weyers (2002)
	CTX110	Tc^R mini CTX1 cloning vector with a *gfp*(LAA) cassette cloned on a *Spe*I RBSII *gfp*(LAA) T_0T_1 *Not*I fragment from pBS110	This study
	CTX111	Tc^R mini CTX1 cloning vector with a *gfp*(LVA) cassette cloned from on a *Spe*I RBSII *gfp*(LVA) T_0T_1 *Not*I fragment from pBS111	This study
	CTX112	Tc^R mini CTX1 cloning vector with a *gfp*(AAV) cassette cloned on a *Spe*I RBSII *gfp*(AAV) T_0T_1 *Not*I fragment from pBS112	This study

	Relevant genotype or phenotype	Source or reference
CTX113	Tc^R mini CTX1 cloning vector with a *gfp*(ASV) cassette cloned on a *Spe*I RBSII *gfp*(ASV) T_0T_1 *Not*I fragment from pBS113	This study
mini CTX1	Tc^R self proficient integration vector with *tet* Ω *FRT attP* MCS *ori int* and *oriT*	Hoang *et al* (2000)
pBluescript® SK ( )	Ap^R ColE1 origin *lacZ* MCS GenBank accession number 52324	Stratagene
pBS3B	Ap^R pBluescript cloning vector with a *gfp*mut3 cassette in a *Xba*I RBSII *gfp*mut3 T_0T_1 *Not*I fragment from pJBA27	Weyers (2002)
pBS110	Ap^R pBluescript® cloning vector with a *gfp*(LAA) cassette in a *Xba*I RBSII *gfp*(LAA) T_0T_1 *Not*I fragment from pJBA110	Weyers (2002)
pBS111	Ap^R pBluescript cloning vector with a *gfp*(LVA) cassette in a *Xba*I RBSII *gfp*(LVA) T_0T_1 *Not*I fragment from pJBA111	Weyers (2002)
pBS112	Ap^R pBluescript® cloning vector with a *gfp*(AAV) cassette in a *Xba*I RBSII *gfp*(AAV) T_0T_1 *Not*I fragment from pJBA112	Weyers (2002)
pBS113	Ap^R pBluescript cloning vector with a *gfp*(ASV) cassette in a *Xba*I RBSII *gfp*(ASV) T_0T_1 *Not*I fragment from pJBA113	This study
pC3BpRA	Tc^R CTX3B with the *rhlAB* promoter region cloned from pGAP on a *Kpn*I – *Bam*HI fragment	This study
pC113pRA	Tc^R CTX113 with the *rhlAB* promoter region cloned from pGAP on a *Eco*RI – *Bam*HI fragment	This study
pGAP	Ap^R pGEM® T Easy cloning vector containing *rhlAB* promoter region amplified with primers AIF and AIR2	This study
pGEM® T Easy	Ap^R ColE1 replicon TA cloning vector for PCR products	Promega
pFLP2	Ap^R *ori1600 oriT* source of *sacB* 9 3 kb	Hoang *et al* (1998)
pJBA27	Ap^R pUC18Not with $P_{A1/04/03}$ RBSII *gfp*mut3 T_0T_1	Andersen *et al* (1998)
pJBA110	Ap^R pUC18Not with $P_{A1/04/03}$ RBSII *gfp*(LAA) T_0T_1	Andersen *et al* (1998)
pJBA111	Ap^R pUC18Not with $P_{A1/04/03}$ RBSII *gfp*(LVA) T_0T_1	Andersen *et al* (1998)
pJBA112	Ap^R pUC18Not with $P_{A1/04/03}$ RBSII *gfp*(AAV) T_0T_1	Andersen *et al* (1998)
pJBA113	Ap^R pUC18Not with $P_{A1/04/03}$ RBSII *gfp*(ASV) T_0T_1	Andersen *et al* (1998)
pRK600	Cm^R ColE1 replicon RK2 Mob RK2 Tra helper plasmid in triparental conjugations	Kessler *et al* (1992)

		Relevant genotype or phenotype	Source or reference
Primers			
PCR	AIF	5 GGTACCCAGAGCGTTTCGACAC 3	This study
	AIR2	5 GGATCCGAACAGGCAAACAGCTATC 3	This study
Sequencing	M13 Reverse	5 GGAAACAGCTATGACCATG 3	Stratagene
	M13 20	5 GTAAAACGACGGCCAGT 3	Stratagene
	T3	5 AATTAACCCTCACTAAAGGG 3	Strategene

* Underlined are the recognition sequence for *Kpn*I in primer AIF and for *Bam*HI in primer AIR2 The primers were designed to amplify the intergenic region upstream of the *rhlAB* gene containing the *hlAB* promoter sequence

4 2 3 Polymerase chain reaction (PCR)

4 2 3 1 Oligonucleotide primers

Oligonucleotide primers AIF and AIR2 (Table 4 1) were used to amplify the *rhlAB* promoter region from *P aeruginosa* genomic DNA The primers were designed using DNAMAN Version 4 13 (Lynnon Biosoft) and Amplify 1 2 on the basis of the published *P aeruginosa* PAO1 genome sequence (Stover *et al* 2000) available at www pseudomonas com Unique restriction enzyme recognition sites were incorporated at the 5 terminus of each primer to facilitate cloning procedures The specificity of the primer set for its intended target region was verified by BLASTN analysis against the *Pseudomonas aeruginosa* genome sequence and GenBank database (at www ncbi nlm nih gov/BLAST) The primers were synthesised by Inqaba Biotech Pretoria South Africa

4 2 3 2 PCR amplification

Each PCR reaction mixture (50 µl) contained 200 ng template DNA 200 µM of each dNTP 32 pmol primer AIF 54 pmol primer AIR2 5% (v/v) DMSO 7 5 mM $MgCl_2$ 1 x PCR buffer and 1 U of SUPERTHERM DNA polymerase (Southern Cross Biotechnology) The tubes were placed in a Applied Biosystems GeneAmp© PCR System 2400 and following denaturation for 3 min at 94 C the reactions were subjected to 35 cycles of denaturation for 30 s at 94 C annealing for 45 s at 55 C and elongation for 2 min at 72 C After the last cycle the reaction mixtures were kept for 5 min at 72 C to complete synthesis of all strands

4 2 4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook *et al* (1989) Briefly 0 8% (w/v) agarose gels were cast and electrophoresed at 90 V in 1 x TAE buffer (40 mM Tris HCl 2 mM EDTA 20 mM acetic acid pH 8 0) To allow for visualisation of the DNA the gels were supplemented with 1 µg/µl ethidium bromide and viewed on a UV transiluminator (Vilber Lourmat) Gel images were captured using a digital gel documentation system (Vilber Lourmat)

4 2 5 Isolation of DNA fragments from agarose gels

DNA fragments were purified from agarose gels using a silica suspension as described by Boyle and Lew (1995) The DNA fragment of interest was excised from the agarose gel with a scalpel blade weighed and mixed with 1 ml of a 6 M NaI solution per 1 g of agarose gel The agarose was melted by incubation at 55 C after which 10 µl of a silica suspension was added Following incubation at 4 C for 30 min with intermittent vortexing the DNA silica complex was collected by centrifugation for 30 s at 13 000 x *g* and the pellet washed four times with 500 µl ice cold New Wash (50 mM NaCl 10 mM Tris HCl 25 mM EDTA 50% [v/v] ethanol) The DNA was subsequently eluted from the silica matrix at 55 C for 10 min in 25 µl ddH_2O An aliquot of the eluted DNA was analysed by electrophoresis on a 0 8% (w/v) agarose gel to assess the concentration and purity of the DNA

4 2 6 DNA molecular markers

Four DNA molecular weight markers were used throughout the study Excepting for the GeneRuler™ 100 bp DNA ladder Plus which was obtained from Fermentas AB the other three DNA molecular weight markers were prepared by digestion of either λ DNA (Roche) or plasmid DNA with different restriction endonucleases Digestion of phage λ DNA with *Hind*III yielded fragments corresponding in size to 23 130 bp 9 416 bp 6 557 bp 4 361 bp 2 322 bp 2 027 bp and 564 bp Alternatively the phage λ DNA was digested with both *Eco*RI and *Hind*III to yield fragments of 21 226 bp 5 148 bp 4 973 bp 4 268 bp 3 530 bp 2 027 bp 1 904 bp 1 584 bp 1 375 bp 947 bp 831 bp and 564 bp The third DNA molecular weight marker was prepared by digestion of plasmid pFLP2 (Hoang *et al* 1998) with both *Bam*HI and *Hind*III to yield DNA fragments of 4 634 bp 2 623 bp 1 310 bp 605 bp and 125 bp All DNA markers were stored at 4 C

4 2 7 Restriction endonuclease digestions

Restriction enzyme digestions were performed in microfuge tubes containing 200 – 800 ng of plasmid DNA and 5 U of enzyme in the appropriate buffer (using the 10x buffer supplied by the manufacturer) The reaction volumes were typically 20 – 30 µl and either incubated for 1 h or overnight at 37 C Digestion of plasmid DNA with two enzymes requiring different salt concentrations for optimal activity was performed by first digesting with the enzyme requiring a lower salt concentration after which the salt concentration was adjusted and the second enzyme added The restriction endonucleases were supplied by Roche The digestion reaction mixtures were analysed on a 0 8% (w/v) agarose gel and DNA fragments required for cloning were purified from the gel using the procedure described above (Section 4 2 5) Alternatively as in the case of amplicons the digested DNA was precipitated from the reaction mixture by the addition of 0 3 volume of 7 5 M NH_4OAc and 1 volume of isopropanol Following incubation for 1 h or overnight at 4 C the DNA was pelleted by centrifugation at 12 000 x *g* for 10 min rinsed with 70% ethanol vacuum dried and suspended in the appropriate volume of ddH_2O

4 2 8 Cloning of DNA fragments

4 2 8 1 Ligation reactions

Ligation reactions were performed in 1 5 ml microfuge tubes and incubated overnight at 16 C in a circulating water bath Insert and vector DNA with ratios of 3 1 or 8 1 were ligated in 15 µl volumes containing 1 U T4 Ligase (Roche) and 1 x ligation buffer (2 mM Tris HCl 0 1 mM EDTA 0 5 mM dithioerythritol 6 mM KCl 5% [v/v] glycerol pH 7 5) For cloning of PCR amplicons the pGEM® T Easy vector (Promega) was used The molar ratio of insert to vector was determined according to the manufacturer s instructions The ligation reaction mixtures contained 5 µl of a 2 x Rapid Ligation Buffer 50 ng of pGEM® T Easy vector approximately three fold higher molar concentration of purified amplicon 1 µl T4 DNA Ligase (3 Weiss units/µl) and ddH_2O to a final volume of 10 µl The ligation reactions were incubated overnight at 16 C Controls consisting of self ligated vector DNA were also included to determine the yield of background non recombinant clones

4 2 8 2 Preparation of competent E coli DH5α cells

Competent *E coli* DH5α cells were prepared according to the method of Inoue as described by Sambrook and Russel (2001) Briefly a single colony of a freshly streaked culture of *E coli* DH5α

was inoculated into 25 ml of SOB broth in a 250 ml Erlenmeyer flask (starter culture) and incubated for 6 – 8 h at 37 C with vigorous shaking (250 – 300 rpm) Different volumes (2 4 and 10 ml) of the starter culture were subsequently inoculated into 1 L Erlenmeyer flasks each containing 250 ml of SOB broth and incubated overnight at 18 – 22 C with moderate shaking The next morning the OD_{600} was measured until a culture had grown to an OD of 0 55 The flask was then transferred to an ice water bath and after incubation for 10 min the cells were collected by centrifugation at 2 500 x *g* for 10 min at 4 C The cell pellet was suspended in 80 ml of filter sterilised ice cold transformation buffer (55 mM $MnCl_2$ $4H_2O$ 15 mM $CaCl_2$ $2H_2O$ 250 mM KCl 10 mM PIPES [pH 6 7]) The cells were collected by centrifugation as above and resuspended in 20 ml transformation buffer followed by the addition of 1 5 ml DMSO After incubation on ice for 10 min the cell suspension was aliquoted into microfuge tubes snap frozen in liquid nitrogen and stored at 70 C

4 2 8 3 Transformation of competent cells

The competent cells were allowed to thaw on ice for 10 min The cells and ligation reaction mixture (not more than 5% of the volume of the competent cells) was mixed and then incubated on ice for 30 min For transformation the cells were subjected to a heat shock at 42 C for 90 s and then immediately placed on ice for 2 min Following incubation 800 µl SOC broth was added and the tubes were incubated for 45 min at 37 C before plating aliquots (100 µl) on selective LB agar plates For selection of transformants based on the tetracycline resistance marker the cells were centrifuged for 2 min at 13 000 x *g* and then resuspended in 100 µl of sterile SOC broth before plating the total volume As a control competent cells alone were plated on the selective agar plate to check for contamination of the competent cells

4 2 9 Plasmid DNA isolation

Plasmid DNA was isolated using a modified alkaline lysis method (Sambrook *et al* 1989) Colonies were picked from the agar plates with sterile toothpicks inoculated into 5 ml LB broth containing the appropriate antibiotic and then incubated overnight at 37 C with shaking Cells from 1 5 ml of the overnight culture were harvested by centrifugation for 2 min at 13 000 x *g* The cell pellet was suspended in 100 µl Solution 1 (50 mM Tris HCl 10 mM EDTA pH 8 0) and incubated at room temperature for 5 min followed by 1 min on ice The cells were lysed by the addition of 200 µl Solution 2 (200 mM NaOH 1% [v/v] SDS) and after incubation on ice for 5 min 300 µl Solution 3 (2 80 M KAc pH 5 1) was added and incubation was continued for 10 min The cellular

debris was collected by centrifugation at 13 000 x *g* for 15 min after which the supernatant was recovered and plasmid DNA precipitated by the addition of 800 µl ice cold 96% ethanol After incubation at 70 C for 30 min the precipitated DNA was collected by centrifugation at 13 000 x *g* for 15 min rinsed with 70% ethanol vacuum dried and suspended in 20 – 30 µl ddH_2O To remove contaminating RNA the plasmid DNA was incubated with 1 µl RNase A (10 mg/ml) for 30 min at 37 C

4 2 10 Nucleic acid sequencing and analysis

The nucleotide sequence of cloned insert DNA was determined using 3 2 pmol of sequencing primer (Table 4 1) and the BigDye™ Terminator v3 1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems) with an ABI PRISM 377 DNA Sequencer or an ABI PRISM 3100 Genetic Analyzer as described previously (Section 3 2 11) Nucleotide sequences were edited using BioEdit Sequence Alignment Editor V5 0 9 (Hall 1999) and their identity verified by BLASTN searches against the GenBank database (at www ncbi nlm nih gov/BLAST) and the *P aeruginosa* genome sequence (at www pseudomonas com)

4 2 11 Construction of mini CTX *gfp* reporter plasmids

To allow investigation of *rhlAB* promoter activity at single cell level in different *P aeruginosa* mutant strains *rhlAB* promoter *gfp* reporter vector constructs were prepared which could be stably integrated into the genome of the respective *P aeruginosa* strains using the mini CTX vector (Hoang *et al* 2000) The strategy used for the construction of the reporter plasmids is indicated in Fig 4 2A All molecular cloning techniques used in the construction of the different reporter plasmids were performed in accordance with the procedures described in the preceding sections

The 450 bp *rhlAB* promoter obtained by PCR amplification using genomic DNA from *P aeruginosa* PAO1 as template (Section 4 2 3) was purified from an agarose gel and cloned into pGEM® T Easy (Promega) to yield recombinant plasmid pGAP Promoterless genes encoding for stable and unstable GFP variants were recovered from the pJBA series of plasmids (Table 4 1 Andersen *et al* 1998) by digestion with both *Xba*I and *Not*I gel purified and then cloned into identically prepared pBluescript® SK( ) vector DNA These intermediate vector constructs and plasmid pGAP were used as the sources to construct the desired mini CTX GFP reporter plasmids To enable cloning of the promoterless *gfp* genes into mini CTX the insert DNA was recovered from the recombinant pBluescript® SK( ) plasmid DNA by digestion with both *Spe*I and *Not*I gel

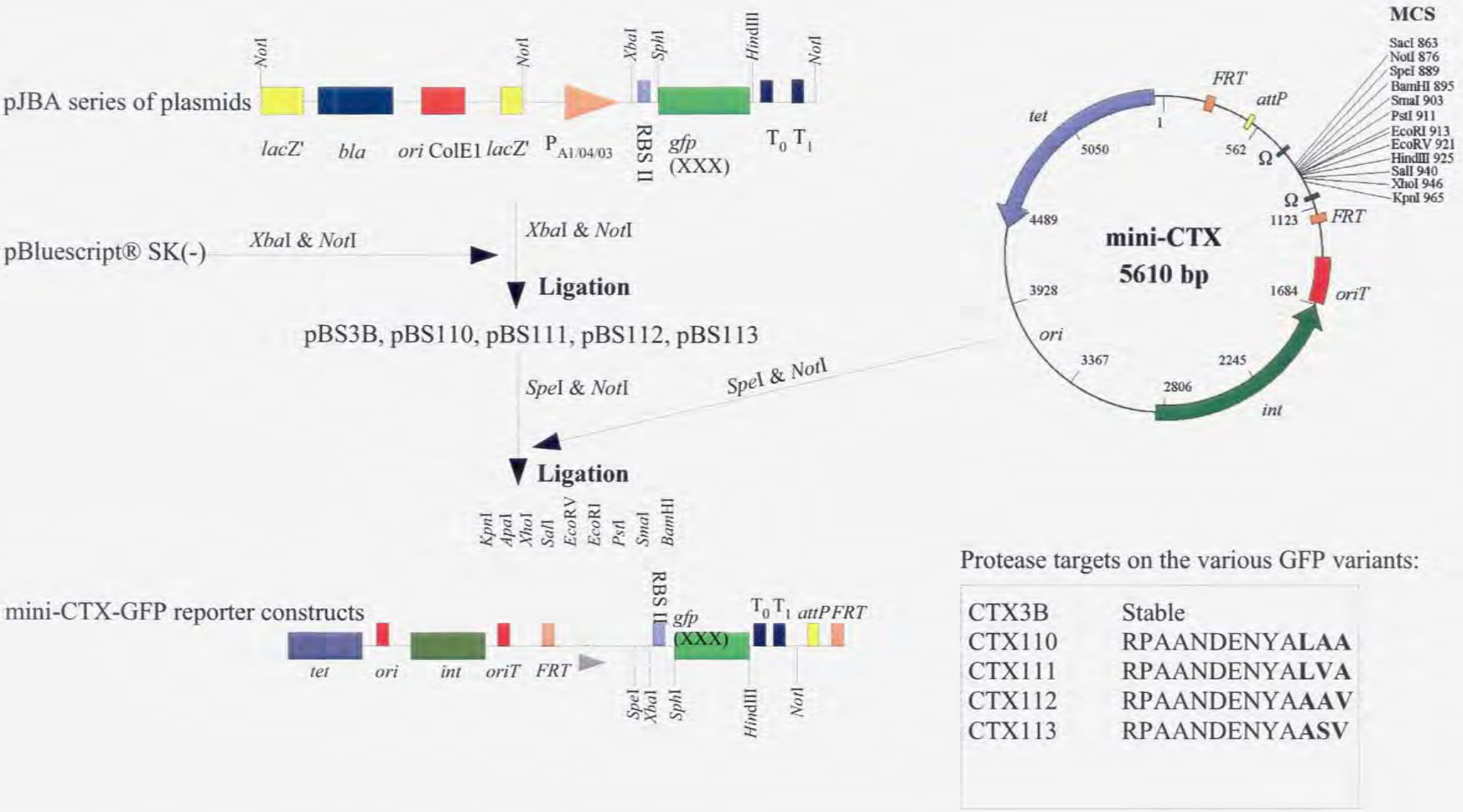
purified and cloned into similarly digested mini CTX The *rhlAB* promoter DNA fragment from plasmid pGAP was cloned into the recombinant mini CTX constructs as either a *Kpn*I – *Bam*HI or *Eco*RI – *Bam*HI fragment upstream from the *gfp* genes to yield pC3BpRA and pC113pRA respectively (Fig 4 2B)

4 2 12 Transformation of *P aeruginosa* strains by triparental mating

Plasmids pC3BpRA and pC113pRA were introduced into *P aeruginosa* PAO1 and mutant strains by triparental conjugation The triparental matings were performed with helper plasmid pRK600 as previously described (Figurski and Helinski 1979) with the following modifications A single colony of freshly streaked cultures of donor (*E coli* DH5α containing recombinant mini CTX vector DNA) recipient (*P aeruginosa* wild type or mutant strains) and helper (*E coli* DH5α containing pRK600) strains were mixed on a LB agar plate with a sterile inoculation loop and then incubated overnight at 37 C Following incubation the mixed growth was streaked on an LB agar plate containing the appropriate antibiotics and incubation was continued for 24 to 48 h at 37 C The resulting recombinant *P aeruginosa* strains were used in further analyses

4 2 13 Fluorometric assays

Glycerol cultures of the recombinant *P aeruginosa* wild type and mutant strains were inoculated into 5 ml low salt LB broth (LB broth containing 0 4% [w/v] NaCl) supplemented with 30 µg/ml tetracycline Following incubation overnight at 37 C 100 µl of the culture was inoculated into 4 ml ABT Glucose medium (ABT medium containing 0 5% [w/v] glucose) in 28 ml McCartney bottles with or without glass wool (0 1 g mean diameter 15 µm Merck) The cultures were incubated for 24 h at 37 C with shaking (200 rpm) Planktonic cells were obtained from cultures grown in the absence of glass wool whilst cultures grown in the presence of glass wool were used as a source of biofilm cells The supernatant of the latter cultures was carefully aspirated Following careful rinsing of the glass wool with sterile medium 4 ml of ABT Glucose medium was added to the glass wool together with 2 g of glass beads (6 mm diameter) and the samples were shaken vigorously for 5 min to remove the biofilm biomass from the glass wool The supernatants were recovered and transferred to new microfuge tubes Fluorescence was measured using a BioRad VersaFluor™ fluorometer equipped with a 480/20 filter for excitation and a 510/10 filter for emission The cell density of the cultures was measured using a SPECTRONIC® 20 GENESYS spectrophotometer at 590 nm against sterile ABT Glucose medium Relative fluorescence was calculated for each culture by dividing the fluorescence value by the cell density value Assays were performed in duplicate



Protease targets on the various GFP variants:

CTX3B	Stable
CTX110	RPAANDENYA**LAA**
CTX111	RPAANDENYA**LVA**
CTX112	RPAANDENYA**AAV**
CTX113	RPAANDENYA**ASV**

Fig. 4.2A Strategy used for the construction of the mini-CTX reporter vector constructs: CTX3B, CTX110, CTX111, CTX112 and CTX113. The gray arrow on the mini-CTX-xxx construct indicates the orientation of the cloned promoter.

Fig. 4.2B Strategy used for the construction of pGAP, pC3BpRA and pC113pRA.

The recombinant *P aeruginosa* wild type and mutant strains were streaked on a low salt LB agar plate and incubated overnight at 37 C Following incubation a single colony of the respective strains was inoculated into 5 ml of low salt LB broth supplemented with 30 µg/ml tetracycline in 28 ml McCartney bottles The cultures were incubated for 4 h at 37 C with shaking (200 rpm) To study *rhlAB* promoter activity during biofilm development biofilms were grown at 37 C in a flow cell with individual dimensions of 2 x 2 x 15 mm supplied with ABT minimal medium (Section 4 2 1) The substratum consisted of a microscope glass coverslip The exponentially growing cultures were injected into separate channels using a 2 ml syringe with a 0 6 x 30 mm gauge needle After inoculation the flow channels were left for 1 h at 37 C and the flow was then switched on at 25 rpm delivering a flow speed of 0 4 mm/s using a Watson Marlow 205S peristaltic pump Images were randomly acquired after 2 h post inoculation and then at 24 h intervals for 72 h using a Zeiss Axiovert 200 fluorescent microscope fitted with a 63x/1 4 Zeiss Neofluor objective and the no 10 Zeiss filter set (excitation 450 to 490 nm emission 525 to 565 nm) The images were captured using a Nikon DXM 1200 digital camera

4 3 RESULTS

4 3 1 Construction of mini-CTX *gfp* reporter vector constructs

To investigate *rhlAB* promoter activity in *P aeruginosa* wild type and mutant strains during biofilm development and maturation reporter plasmids were constructed using mini CTX as vector and both stable and unstable variants of *gfp* as reporter gene (Figs 4 2A and 4 2B) Unstable GFP variants were included in this study since the extreme stability of GFP may be problematic in studies regarding temporal changes in gene expression (Tombolini *et al* 1997) In contrast unstable GFP variants are more susceptible to degradation by ClpXP type proteases and therefore have shorter half lives (Andersen *et al* 1998) The mini CTX cloning system (Hoang *et al* 2000) allows for the stable integration of a single copy of the promoter *gfp* transcriptional fusion into the *attB* site of the *P aeruginosa* genome In addition the mini CTX vector contains a pMB1 derived origin of replication that permits replication in various different gram negative bacteria an *oriT* that permits conjugation mediated plasmid transfer a tetracycline resistance marker gene and a multiple cloning site flanked by T4 transcriptional terminators to reduce basal levels of reporter gene expression

4 3 1 1 Cloning of promoterless gfp genes into mini CTX

The stable and unstable variants of the GFP encoding genes have been cloned previously under transcriptional control of the $P_{A1/04/03}$ promoter in the pJBA series of plasmids (Table 4 1 Andersen *et al* 1998) To obtain promoterless copies of these reporter genes the respective cassettes were recovered by digestion with both *Xba*I and *Not*I and first cloned into identically prepared pBluescript® SK( ) vector DNA before being cloned as *Spe*I – *Not*I DNA fragments into mini CTX vector DNA (Fig 4 2A) The intermediate cloning step into pBluescript® SK( ) was necessary in order to retain most of the restriction endonuclease sites in the multiple cloning site (MCS) of the mini CTX vector Following transformation of competent *E coli* DH5α cells with the ligation reaction mixtures plasmid DNA was extracted from the tetracycline resistant transformants and characterised by restriction enzyme digestion Digestion of the recombinant plasmid DNA with *Hind*III,which cuts once in the MCS of the vector and once at the 3 end of the cloned DNA fragment resulted in digestion products of which the sizes corresponded to the expected size of the mini CTX vector DNA (5 6 kb) and the size of the cloned RBSII *gfp* T_0T_1 cassette (1 2 kb) respectively (Fig 4 3) Two recombinant clones designated CTX3B which contained a promoterless stable GFP variant and CTX113 which contained a promoterless unstable GFP variant (ASV) were selected and used in subsequent plasmid DNA constructions The integrity of the cloned DNA was confirmed by nucleotide sequence analysis using a T3 sequencing primer (Table 4 1)

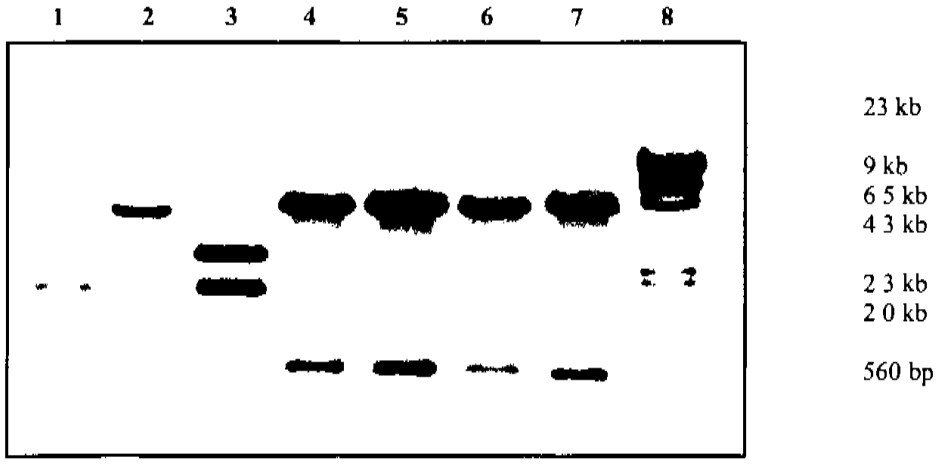


Fig 4 3 Agarose gel electrophoretic analysis of recombinant mini CTX vector constructs containing GFP variants Lane 1 agarose gel purified GFP DNA fragment (1 8 kb) lane 2 plasmid CTX3B digested with both *Sph*I and *Not*I lane 3 plasmid pJBA68 digested with both *Sph*I and *Not*I lane 4 CTX110 digested with *Hind*III lane 5 CTX111 digested with *Hind*III lane 6 CTX112 digested with *Hind*III lane 7 CTX113 digested with *Hind*III lane 8 DNA molecular marker The sizes of the DNA molecular weight marker phage λ digested with *Hind*III are indicated to the right of the figure

4 3 1 2 Cloning of the P aeruginosa rhlAB promoter region

To obtain the *rhlAB* promoter oligonucleotide primers AIF (containing a *Kpn*I site) and AIR2 (containing a *Bam*HI site) were used in a PCR with genomic DNA of *P aeruginosa* PAO1 as described under Materials and Methods (Section 4 2 3) An aliquot of the reaction mixture was analysed by agarose gel electrophoresis and a DNA fragment of approximately 430 bp was observed (Fig 4 4 lane 1) The agarose gel purified amplicon was subsequently cloned into pGEM® T Easy vector DNA and restriction of the derived recombinant plasmids with both *Bam*HI and *Kpn*I resulted in the excision of a 430 bp DNA fragment (Fig 4 4 lane 2) indicating that the amplicon had been cloned successfully A recombinant clone designated pGAP was selected and the integrity of the cloned insert DNA was verified by nucleotide sequence analysis using the universal M13/pUC forward and reverse sequencing primers This recombinant clone was used in all subsequent DNA manipulations

4 3 1 3 Construction of rhlAB gfp transcriptional fusions in mini CTX vectors

The recombinant plasmids pGAP CTX3B and CTX113 were used as sources for construction of the desired reporter vectors To construct the reporter plasmid pC3BpRA the *rhlAB* promoter DNA fragment was recovered from pGAP by digestion with both *Bam*HI and *Kpn*I and cloned directionally into similarly prepared plasmid CTX3B whereas reporter plasmid pC113pRA was constructed by cloning the *rhlAB* promoter as a *Bam*HI – *Eco*RI DNA fragment into CTX113 (Fig 4 2B) This was necessary as *Kpn*I had multiple recognition sites within the CTX113 plasmid that may have been introduced into the *gfp* (ASV) cassette during the original construction of the unstable GFP variant The recombinant plasmid DNA was subsequently characterised by restriction enzyme digestion Digestion of pC3BpRA with *Bam*HI and *Kpn*I (Fig 4 4 lane 3) and pC113pRA with *Bam*HI and *Eco*RI (Fig 4 4 lane 4) yielded DNA fragments corresponding to the size of the mini CTX GFP vector DNA (6 4 kb) and the size of the cloned *rhlAB* promoter (430 bp)

In summary two reporter vectors have thus been constructed Plasmid pC3BpRA contained a *rhlAB gfp*mut3 transcriptional fusion that encodes a very stable high fluorescent intensity GFP variant (Cormack *et al* 1996) In contrast pC113pRA contained a *rhlAB gfp*mut(ASV) transcriptional fusion that encodes a GFP variant that has a half live of 2 h in *P aeruginosa* and is particularly suited to analysis of weak promoters such as the *rhlAB* promoter (Pers comm Hentzer 2003) Although other GFP variants are available with much shorter half lives (up to 40 min) they are better suited to the study of strong promoters and were excluded from use in this study

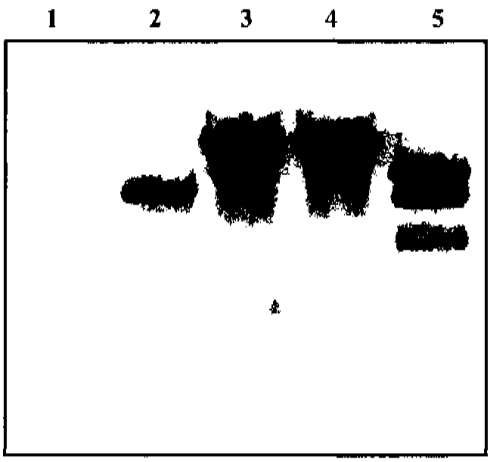


Fig 4 4 Agarose gel electrophoretic analysis of the recombinant mini CTX GFP reporter vectors Lane 1 PCR amplified *rhlAB* promoter region (430 bp) lane 2 plasmid pGAP digested with both *Bam*HI and *Kpn*I lane 3 reporter vector pC3BpRA digested with both *Bam*HI and *Kpn*I lane 4 reporter vector pC113pRA digested with both *Eco*RI and *Bam*HI lane 5 DNA molecular marker The sizes of the DNA molecular weight marker plasmid pFLP2 digested with *Bam*HI and *Hin*dIII are indicated to the right of the figure

4 3 2 Generation of recombinant *P aeruginosa* strains containing an integrated copy of the reporter plasmids

Strains containing an integrated copy of the reporter vector constructs were generated by introducing plasmids pC3BpRA and pC113pRA into different *P aeruginosa* strains by conjugation and then selecting for subsequent single recombination events using a selective medium as described under Materials and Methods (Section 4 2 12) The recipient strains used were the wild type PAO1 strain PAO1Δ*rpoN* (an RpoN deficient PAO1 strain) and SS24 (an RpoS deficient PAO1 strain) The resultant recombinant strains were designated PA3B PA113 N3B N113 S3B and S113 respectively where 3B denotes the stable GFP variant and 113 denotes the unstable GFP variant The integrants were used in all subsequent investigations and cultured in medium supplemented with 30 µg/ml tetracycline to maintain the chromosomal insertions in the different recombinant strains

4 3 3 Assaying of *rhlAB* promoter activity

4 3 3 1 rhlAB promoter activity in batch culture

To investigate *rhlAB* promoter activity in the planktonic and biofilm microcosms of wild type *P aeruginosa* PAO1 and mutant strains lacking either the RpoS or RpoN sigma factors the

recombinant wild-type and mutant *P. aeruginosa* strains were assayed for GFP expression by fluorometry. Non-recombinant strains were included in these assays as controls to determine the level of autofluorescence often associated with *P. aeruginosa*. The results were analysed as described under Materials and Methods (Section 4.2.13) and are presented in Fig. 4.5. For these assays, fluorescence measurements were taken after 24 h of incubation, which coincided with the presence of well-developed biofilms on the glass wool and transcriptionally active *rhlAB* promoter, as verified by analysis of samples from the batch cultures by bright-field and fluorescent microscopy, respectively.

Analyses of the results indicated that GFP expression was up-regulated in the biofilm population of the wild-type PAO1 (PA3B) and the RpoS-deficient SS24 (S3B) strains by as much as 50% and 310%, respectively, when compared to the corresponding planktonic populations. However, reporter gene expression was similar in the planktonic and biofilm populations of the RpoN-deficient strain N3B. Reporter gene expression from S3B was down-regulated in both planktonic (62%) and biofilm (26%) populations when compared to the corresponding populations of PA3B. In contrast, reporter gene expression from N3B was up-regulated by 30% in the planktonic population and down-regulated by 13% in the biofilm population when compared to that of PA3B. Similar results were obtained for recombinant strains expressing the unstable GFP variant as reporter, excepting that the relative fluorescence values were lower, probably as a consequence of its shorter half-life.

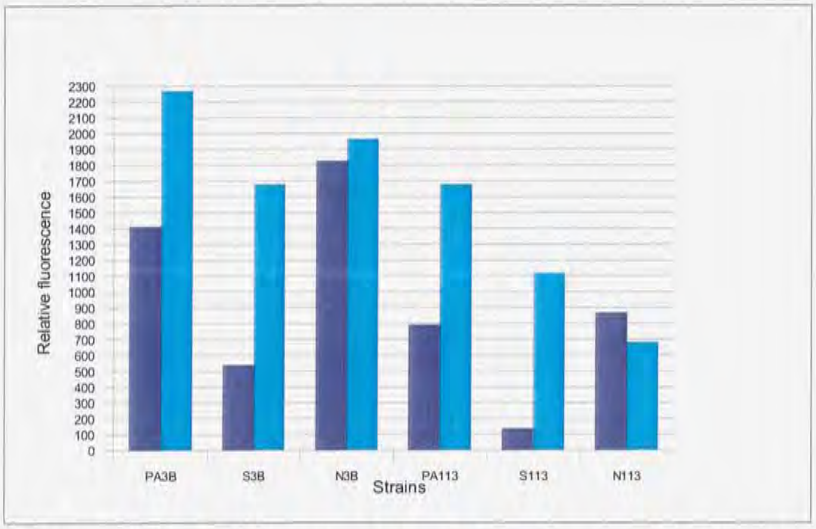


Fig. 4.5 Graph displaying the relative fluorescence per total cellular biomass of the different *rhlAB-gfp* reporter constructs in planktonic (in purple) and biofilm (in blue) populations of wild-type PAO1 (PA3B and PA113), RpoS-deficient (S3B andS113) and RpoN-deficient (N3B and N113) *P. aeruginosa* mutant strains.

4 3 3 2 rhlAB promoter activity in biofilms under conditions of continuous flow

To investigate the transcriptional activity of the *rhlAB* promoter in single cells during biofilm development the different recombinant *P aeruginosa* strains harbouring an integrated copy of the transcriptional fusions between the *rhlAB* promoter and either the stable or unstable *gfp* variants were inoculated into a flow cell and GFP expression was monitored by epifluorescence microscopy The results of these investigations are indicated in Figs 4 6 through 4 11 Generally the cells of strains tagged with the unstable GFP variant (Figs 4 7 4 9 4 11) displayed less fluorescence compared to the cells of corresponding strains tagged with the stable GFP protein (Figs 4 6 4 8 4 10) This may have been due to the higher susceptibility of the unstable GFP variant to degradation by ClpXP type proteases

P aeruginosa PAO1 cells were observed attaching to specific areas on the surface as early as 2 h after inoculation and these cells subsequently coalesced to form localised microcolonies The microcolonies then developed to form structured biofilms interspersed by channels (Figs 4 6 and 4 7) Although no fluorescent cells could be seen after 2 h microcolonies however fluoresced brightly and single bright cells could be seen around the microcolonies The fluorescence did not appear to be localised to specific areas within the microcolonies and was heterogeneous with some cells and cell clusters in the biofilm fluorescing brighter than the rest of the cells

The biofilm formed by the RpoS deficient *P aeruginosa* strain (Figs 4 8 and 4 9) was distinctly different to that of the wild type PAO1 strain The cells of the *rpoS* mutant attached indiscriminately to the surface 2 h after inoculation The biofilms appeared as thick flat mats with no visible channels No cells could be seen fluorescing 2 h after inoculation but the whole biofilm fluoresced brightly and the fluorescence did not appear to be localised to specific areas or planes within the undifferentiated biofilm

The biofilm formed by the RpoN deficient *P aeruginosa* strain (Figs 4 10 and 4 11) more closely resembled those of the wild type PAO1 strain Single cells that attached randomly 2 h after inoculation started to aggregate into microcolonies after 24 h which then developed into well defined microcolonies separated by dark fluid filled channels The microcolonies were on average three to four times larger than microcolonies of the wild type PAO1 strain and they became denser and larger after 24 h No localisation of fluorescence in the biofilm was observed and the entire microcolonies fluoresced brightly

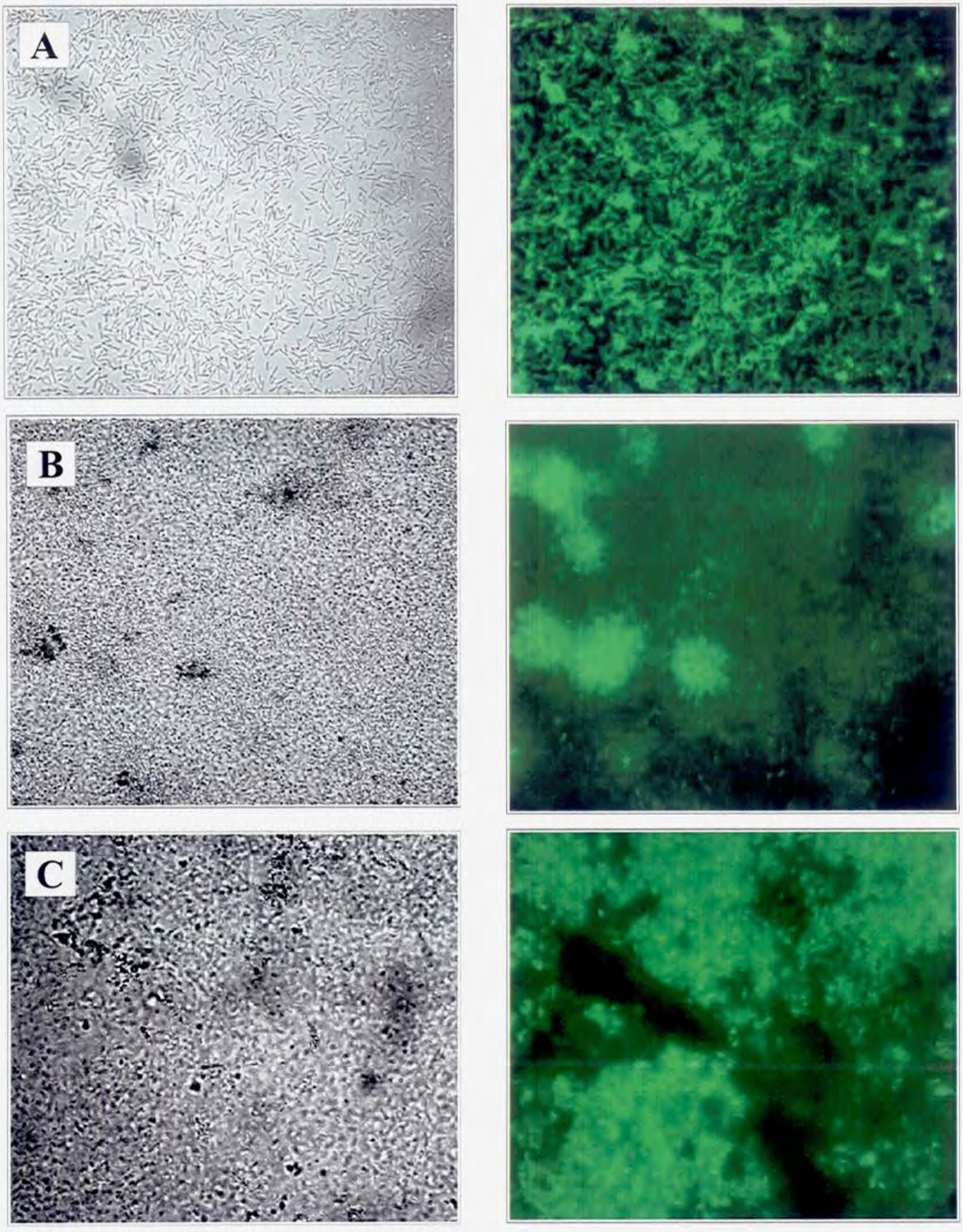


Fig. 4.6 Biofilm structure and *rhlAB* promoter activity of strain PA3B (PAO1 wild-type with *rhlAB::gfp*[stable]) at 24 h (A), 48 h (B) and 72 h (C). The left panel shows bright-field micrographs and the right panel shows epifluorescence micrographs of the corresponding fields. Magnification is 1000x.

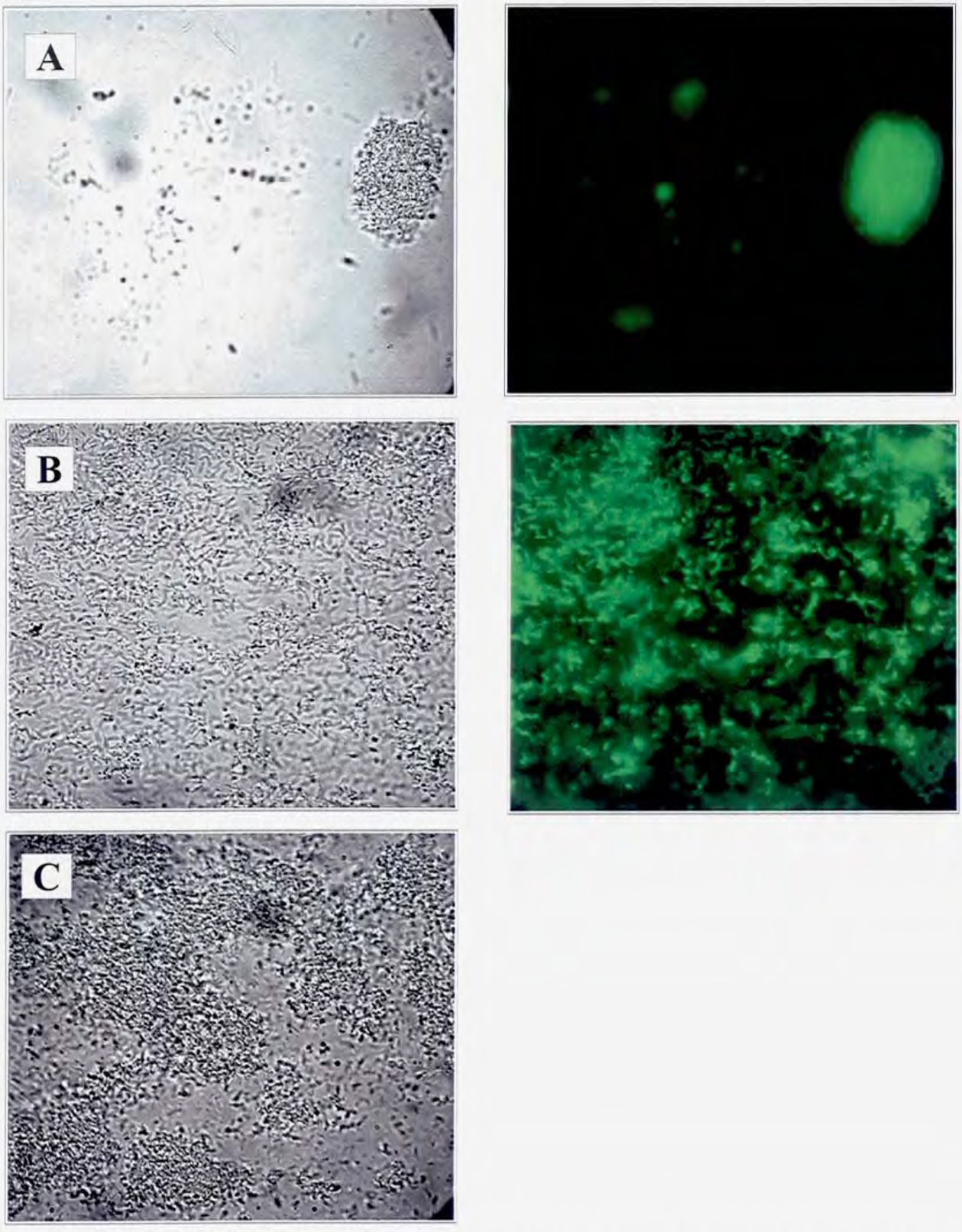


Fig. 4.7 Biofilm structure and *rhlAB* promoter activity of strain PA113 (PAO1 wild-type with *rhlAB::gfp*[unstable]) at 24 h (A), 48 h (B) and 72 h (C). The left panel shows bright-field micrographs and the right panel shows epifluorescence micrographs of the corresponding fields. Magnification is 1000x.

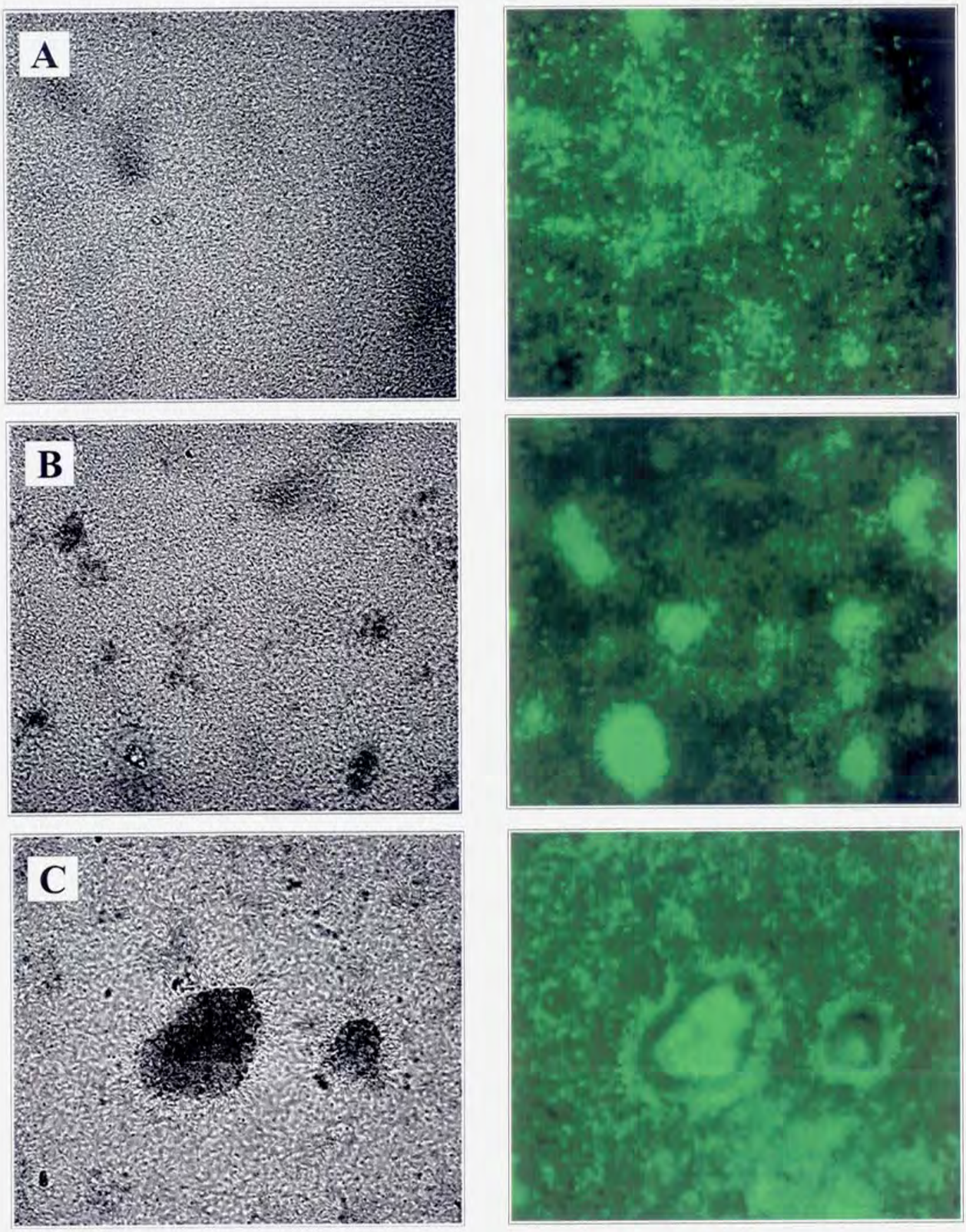


Fig. 4.8 Biofilm structure and *rhlAB* promoter activity of strain S3B (PAO1Δ*rpoS* with *rhlAB::gfp*[stable]) at 24 h (A), 48 h (B) and 72 h (C). The left panel shows bright-field micrographs and the right panel shows epifluorescence micrographs of the corresponding fields. Magnification is 1000x.

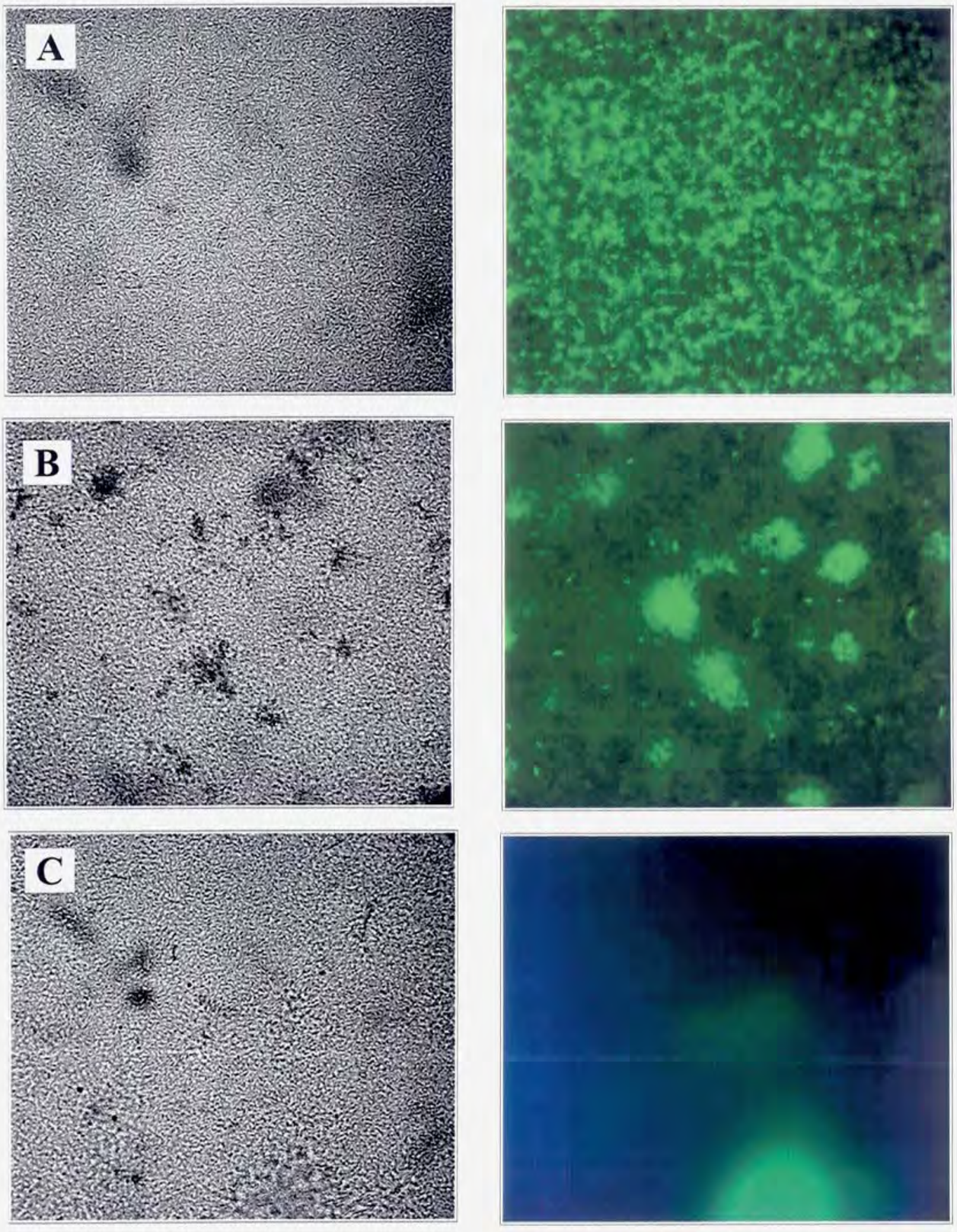


Fig. 4.9 Biofilm structure and *rhlAB* promoter activity of strain S113 (PAO1Δ*rpoS* with *rhlAB::gfp*[unstable]) at 24 h (A), 48 h (B) and 72 h (C). The left panel shows bright-field micrographs and the right panel shows epifluorescence micrographs of the corresponding fields. Magnification is 1000x.

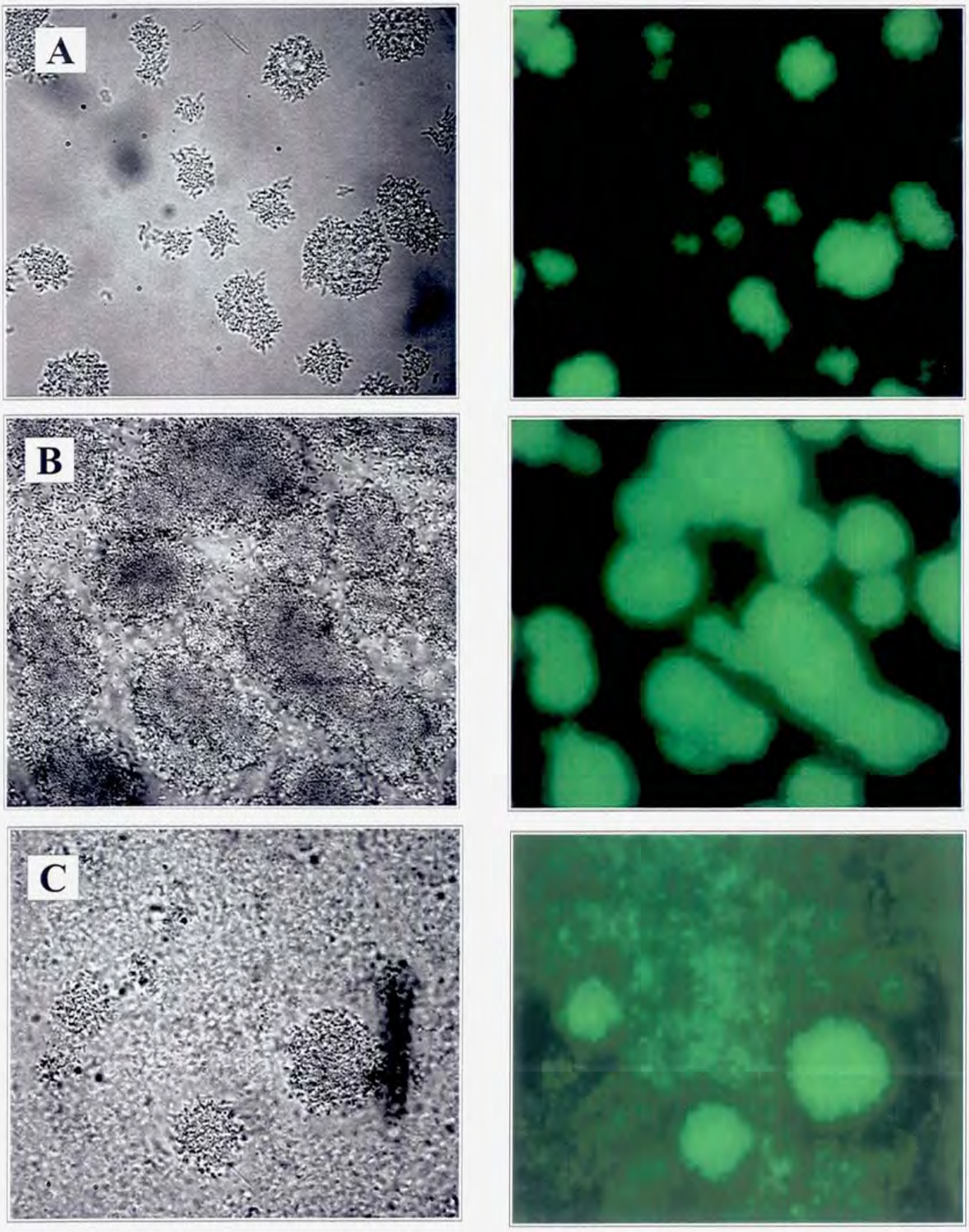


Fig. 4.10 Biofilm structure and *rhlAB* promoter activity of strain N3B (PAO1Δ*rpoN* with *rhlAB::gfp*[stable]) at 24 h (A), 48 h (B) and 72 h (C). The left panel shows bright-field micrographs and the right panel shows epifluorescence micrographs of the corresponding fields. Magnification is 1000x.

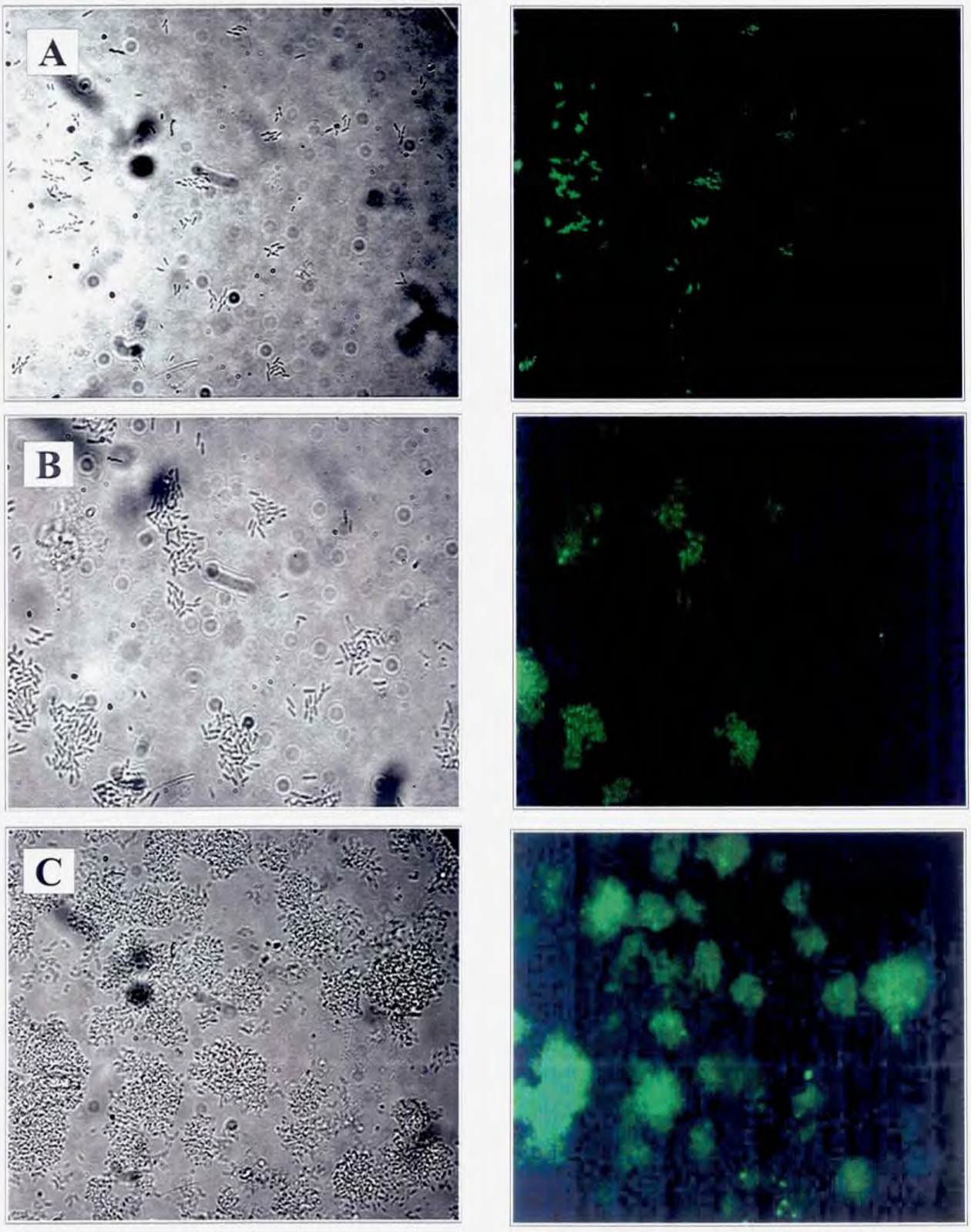


Fig. 4.11 Biofilm structure and *rhlAB* promoter activity of strain N113 (PAO1Δ*rpoN* with *rhlAB::gfp*[unstable]) at 24 h (A), 48 h (B) and 72 h (C). The left panel shows bright-field micrographs and the right panel shows epifluorescence micrographs of the corresponding fields. Magnification is 1000x.

4 4 DISCUSSION

To facilitate studies regarding *rhlAB* promoter activity in *P aeruginosa* wild type PAO1 RpoS and RpoN null mutant strains during biofilm development and maturation several reporter vectors were constructed in this part of the study For this purpose transcriptional fusions between the *P aeruginosa rhlAB* promoter and stable as well as unstable variants of the *gfp* gene were constructed Although plasmid based reporter systems have been used frequently in the study of environmental control of gene expression (Davies *et al* 1993 Sternberg *et al* 1999 de Kievit *et al* 2001) they suffer several disadvantages Notably being plasmid based systems the plasmid copy number may differ between cells and require antibiotic selective pressure to maintain the plasmid in the cell This together with expression of other plasmid borne genes may affect the physiology of the bacterial cells For example up regulation in expression of the reporter gene may be due to biofilm cells being more resistant to antibiotics that could lead to a higher survival rate (more cells) Consequently the up regulation in expression of the reporter gene may not be directly related to an increase in promoter activity Furthermore a difference in plasmid copy number may also result in false up regulation as differences in the number of plasmid copies per cell in a particular growth phase would yield differences in reporter gene activity To overcome these problems mini CTX GFP reporter vectors were rather constructed which allow for integration of a single copy of the transcriptional fusion in a defined non essential region on the *P aeruginosa* genome In addition the use of *gfp* as a reporter of promoter activity allowed for studying the *P aeruginosa* biofilm microcosm in a non invasive manner and without the addition of exogenous substrates or energy sources (Chalfie *et al* 1994)

Wild type and mutant *P aeruginosa* PAO1 strains containing integrated copies of the *rhlAB gfp* transcriptional fusions were cultured in ABT medium in the presence and absence of glass wool as attachment substratum Using a fluorometer to measure the relative fluorescence a general increase in *rhlAB* promoter activity was observed in the biofilm populations compared to the corresponding planktonic populations of each strain (Fig 4 5) However the *rhlAB* promoter activity was lowest in the planktonic and biofilm populations of the RpoS deficient mutant strain whilst similar levels of *rhlAB* promoter activity were observed in planktonic and biofilm populations of an RpoN deficient strain These results suggested that RpoS positively regulates *rhlAB* expression which is in agreement with a report by Medina *et al* (2003b) indicating that *rhlAB* expression is partially RpoS dependent In contrast the relationship between RpoN and *rhlAB* expression has not been

completely resolved Transcription of the *rhlAB* promoter has been proposed to be σ^{54} dependent based on the reduced level of its expression in a *P aeruginosa rpoN* mutant (Pearson *et al* 1997) However Heurlier *et al* (2003) reported that an *rpoN* mutant over expressed the biosynthetic genes for rhamnolipids and a two to four fold increase of *rhlAB* expression was obtained in the mutant strain compared to the wild type *P aeruginosa* PAO1 strain In contrast Medina *et al* (2003a) reported that *rhlAB* expression is RpoN independent and that the transcriptional regulation of *rhlAB* is rather at a σ^{70} dependent promoter which overlaps the putative σ^{54} promoter in the upstream region of the *rhlAB* promoter (Fig 4 1) Consequently Medina and co workers concluded that any up or down regulation in the transcription of the *rhlAB* genes in an RpoN null mutant would be the result of indirect influences possibly via RhlR The *rhlR* gene which encodes the transcriptional regulator RhlR and plays a role in the transcriptional regulation of the *rhlAB* promoter has four different transcriptional start sites of which one start site is σ^{54} dependent (Medina *et al* 2003a) Thus RpoN may regulate expression of RhlR which in turn would influence expression of the *rhlAB* promoter This may explain why *rhlAB* appears to be regulated by RpoN as has been previously observed (Pearson *et al* 1997 Heurlier *et al* 2003) Expression of *rhlR* is subject amongst other to a complex transcriptional regulation that is greatly influenced by media composition In this study ABT medium was used and its influence on expression on RhlR is not known and was also not investigated further in this study Therefore the lack of significant up or down regulation of *rhlAB* promoter activity in the RpoN null mutant compared to the wild type PAO1 strain (Fig 4 5) may be due to RpoN independent transcription of the *rhlAB* promoter as suggested by Medina *et al* (2003a) or due to an indirect influence via the effect of the growth medium used in this study on the transcription of *rhlAB* and *rhlR* (Medina *et al* 2003c)

Since biofilms are heterogeneous assaying of the whole population for reporter activity may not reveal the heterogeneity of expression in a subset of the population as only the average activity will be measured To overcome these limitations a flow cell system was used to investigate *rhlAB* promoter activity during biofilm development The results that were obtained indicated differences in biofilm structures between wild type PAO1 the RpoS null mutant and the RpoN null mutant (Figs 4 6 through 4 11) Both the wild type PAO1 and RpoN null mutant showed distinct microcolonies separated by fluid filled channels but the microcolonies of the mutant was larger than that of the wild type and it displayed significantly wider channels between the microcolonies In contrast the RpoS null mutant formed undifferentiated biofilms without visible channels The biofilm formed by the *rpoS* mutant resembles that of an *rhlA* mutant which has been reported to

produce a biofilm consisting of a thick uniform mat of *P aeruginosa* cells (Davey *et al* 2003) Although the *rhlAB* mutant was able to form channels surrounding microcolonies it was unable to maintain them Consequently it was concluded that rhamnolipid is used to maintain the void spaces surrounding the microcolonies (Davey *et al* 2003) Although not determined in this study a fellow student has previously determined that the concentration of rhamnolipid per unit biomass was approximately three fold lower in biofilms of the *rpoS* mutant (used in this study) compared to a wild type PAO1 strain (Pers comm MacDonald 2003) Therefore the lack of visible channels observed in well developed biofilms of the RpoS null mutant may have been due to the lack of rhamnolipid in the biofilm Thompson *et al* (2003) referring to unpublished data by Davey and O Toole reported that the *rpoN* mutant is deficient in rhamnolipid synthesis If this were indeed the case then the biofilms of the RpoN null mutant would be expected to resemble those of the RpoS null mutant This was however not the case Unfortunately as in the case of Thompson *et al* (2003) the concentration of rhamnolipid per unit biomass in the biofilm population was not determined for the *rpoN* mutant used in this study In addition based on reports by Medina *et al* (2003a c) indicating that transcription of *rhlAB* is RpoN independent and on the results obtained in this study indicating significant levels of *rhlAB* promoter activity in the planktonic and biofilm populations of the *rpoN* mutant (Fig 4 5) the possibility that the *rpoN* mutant used in this study may produce rhamnolipid cannot be excluded Therefore if the RpoN null mutant does indeed produce rhamnolipid then this may explain the maintenance of the channels between microcolonies It was beyond the scope of this study to determine specific phenotypes of the RpoN null mutant that contributed to their changed biofilm morphology i e larger microcolonies and wider channels compared to the wild type PAO1 strain

For the wild type PAO1 and respective mutant strains fluorescent cells were not observed 2 h after attachment of the cells to a surface in a flow through system but transcription of *rhlAB* appeared to be constitutive from 24 h onwards and was not localised to specific areas within the biofilm These results are in agreement with previous reports indicating a lack of transcription of the *rhlAB* promoter early in the biofilm formation process and that rhamnolipid does not play a role in the initial attachment of single cells to a surface (Ochsner *et al* 1994 Davey *et al* 2003) Since binding of RhlR(C_4 HSL) to the *lux* like box in the upstream promoter region of *rhlAB* is a necessary condition for *rhlAB* expression and based on observations that entire microcolonies within the respective biofilms fluoresced brightly the lack of localised transcriptional activation of *rhlAB* suggests the C_4 HSL inducer molecule diffused readily throughout the respective biofilms

CHAPTER 5

CONCLUDING REMARKS

The aims of this study were (i) to develop and optimise a biochemical assay for rhamnolipid extraction and quantitation (ii) to generate a *rhlAB* deficient mutant strain of *P aeruginosa* PAO1 by directed insertional mutagenesis using an appropriate allelic exchange vector and (iii) to investigate the transcriptional activity of the *P aeruginosa rhlAB* promoter in different mutant strains and under different growth conditions using reporter gene technology The information that has evolved during this study are summarised briefly in the following sections

Although various methodologies for the quantification of rhamnolipid exist many of these require the use of specialised equipment (Harkins and Alexander 1959 Deziel *et al* 2000 Gartshore *et al* 2000) However several colorimetric assays have been reported which do not rely on the use of specialised equipment (Zhang and Miller 1992 Deziel *et al* 1996 Sim *et al* 1997) These methods were subsequently used to develop an improved protocol for the quantification of rhamnolipid following its extraction from small sample volumes of a *P aeruginosa* culture (Chapter 2) Although the optimised assay does not rely on the use of specialised equipment and is cost effective it is however time consuming and is unable to distinguish between different structural variants of rhamnolipid In this study the rhamnolipid assay was used to screen putative RhlAB null mutants and it has also been used by a fellow PhD student to determine the rhamnolipid concentration of biofilm and planktonic populations of an RpoS deficient *P aeruginosa* strain

Of the various different approaches and vector systems having been reported whereby targeted mutations can be introduced in the genomes of bacteria (Hayes 2003 Goldberg and Ohman 1987 Schweizer and Hoang 1995 Corbin *et al* 1982 Marx and Lidstrom 2002 Wong and Mekalanos 2000) the use of so called suicide plasmids has remained popular Although this approach has been used with success in our laboratory to construct various mutant *P aeruginosa* strains it was found in this study that *rhlAB* was not stably maintained in ColE1 based vectors which are conditional for replication in *P aeruginosa* Since no other vector systems for generating targeted mutations in the *P aeruginosa* genome were available different broad host range mobilisable allelic exchange vectors were constructed and evaluated for their ability to introduce site specific mutations into the *P aeruginosa* genome (Chapter 3) Of the different vectors and approaches used in this study to inactivate the *P aeruginosa rhlAB* genes the most efficient was based on the use of two incompatible plasmids each of which harboured a counter selectable *sacB* marker gene By making use of the allelic exchange vector pPTSac ABK together with the chase plasmid pJBSac approximately 90% of the resultant *rhlAB* mutant strains appeared to be double reciprocal mutants

Several of these *rhlAB* mutant strains were subsequently shown to be deficient in rhamnolipid synthesis using the newly developed and optimised rhamnolipid assay The *rhlAB* mutant strain was to be used in studies regarding the importance of rhamnolipid in maintaining *P aeruginosa* biofilm architecture However just prior to undertaking these investigations a paper by Davey *et al* (2003) appeared indicating that rhamnolipid plays a role in maintaining the channels between microcolonies Thus the *rhlAB* mutant strain was not used further in this study and emphasis was rather shifted towards investigating *rhlAB* promoter activity during biofilm development by *P aeruginosa*

In addition to various different global regulators that have been implicated in the direct or indirect regulation of *rhlAB* transcription and rhamnolipid production Heurlier *et al* (2003) recently reported that RpoN exerts a global negative control on the quorum sensing machinery of *P aeruginosa* Furthermore RpoS has been implicated in the regulation of various exoproducts (Suh *et al* 1999) and quorum sensing (Whiteley *et al* 2000) Therefore both RpoN and RpoS deficient *P aeruginosa* strains were included in the study to determine the effect of these sigma factors on *rhlAB* transcription during biofilm development To facilitate studies regarding *rhlAB* promoter activity in the *P aeruginosa* wild type PAO1 and respective mutant strains during biofilm development and maturation several mini CTX based reporter vectors were constructed that harboured transcriptional fusions between the *P aeruginosa rhlAB* promoter and stable as well as unstable variants of the *gfp* gene (Chapter 4) Using the wild type and mutant *P aeruginosa* strains containing an integrated copy the *rhlAB gfp* transcriptional fusion *rhlAB* promoter activity was investigated in both a batch and flow through system Results from the batch analysis indicated that RpoS positively regulates *rlhAB* transcription whilst RpoN did not appear to influence *rhlAB* promoter activity to any great extent under the conditions used in this study In contrast to reports by Pearson *et al* (1997) and Heurlier *et al* (2003) these results appear to be in agreement with that of Medina *et al* (2003a) whom reported that transcription of *rhlAB* is RpoN independent However RpoN could indirectly influence the transcription of *rhlAB* by positively regulating Vfr (Thompson *et al* 2003) or via RhlR (Medina *et al* 2003c)

To investigate the heterogeneity in expression of the *rhlAB* promoter in the biofilm population during the development of biofilms by the *P aeruginosa* wild type PAO1 and RpoS and RpoN null mutant strains expression of the *gfp* reporter gene was monitored by fluorescent microscopy over time in a continuous flow through system (Chapter 4) Whereas the *rpoS* mutant strain formed

thick flat mats with no visible channels both the wild type PAO1 and *rpoN* mutant strains formed biofilms interspersed with channels albeit that the channels were wider for the *rpoN* mutant compared to those of the wild type PAO1 strain The transcription of *rhlAB* appeared to be constitutive from 24 h onwards and did not appear to be localised to specific areas within the microcolonies or biofilms indicating that the C_4 HSL inducer molecule which when complexed with RhlR leads to transcription of *rhlAB* was synthesised by the respective strains and diffused readily throughout the biofilm structures Since Davey *et al* (2003) reported that rhamnolipid is important for maintaining the channels and based on the lack of rhamnolipid production in the *rpoS* mutant strain it may be that the absence of channels in biofilms of the *rpoS* mutant strain are due to a lack of rhamnolipid This is in agreement with results obtained in batch assays indicating that RpoS positively regulates *rhlAB* transcription Although rhamnolipid production by the RpoN null mutant strain was not determined in this study Thompson *et al* (2003) referring to unpublished data by Davey and O Toole suggested that an RpoN deficient *P aeruginosa* PAO1 strain does not produce rhamnolipid This would imply that biofilms of the mutant strain should therefore also be devoid of channels which was not the case However it has been reported that transcription of *rhlAB* is RpoN independent whilst RpoN dependent expression of *rhlR* is regulated by environmental factors including culture medium composition (Medina *et al* 2003a b c) Therefore the possibility that rhamnolipid was produced under the conditions used in this study cannot be excluded and corresponds with earlier results obtained in batch assays indicating a high level of *rhlAB* promoter activity in the biofilm population of the RpoN deficient strain

In conclusion during the course of this study a cost effective biochemical assay for the extraction and quantification of rhamnolipid from small sample volumes has been developed and a vector system consisting of pPTSac and pJBSac has been constructed and was shown to be highly efficient in generating *rhlAB* mutant strains of *P aeruginosa* PAO1 Furthermore reporter vector systems utilising stable and unstable variants of *gfp* as reporter gene has been constructed and used to investigate *rhlAB* promoter activity These different tools have already found application in the research projects performed by different members of our research group and have been used with great success Regarding the role and regulation of *rhlAB* transcription during biofilm development this study has highlighted that much research is still needed to clarify the intricate regulatory cascades involved in rhamnolipid biosynthesis in *P aeruginosa* Future research should thus focus on determining the interaction of various global regulators such as Vfr RpoN RpoS and GacA on the transcription and expression of quorum sensing regulated genes including *rhlAB* By

investigating *rhlAB* gene transcription in single and/or multiple knockout strains (targeted against global regulators) effects caused by deletion of the respective regulators on gene transcription can be clarified

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