The role of AlgK in alginate biosynthesis by *Pseudomonas aeruginosa*

A thesis presented in partial fulfilment of the
requirements of the degree of
Master of Science
in
Microbiology
at Massey University, Palmerston North,
New Zealand

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

Yajie Wang
2013

ABSTRACT

Alginate is a polysaccharide produced by brown seaweeds and two bacterial genera *Azotobacter* and *Pseudomonas*. While seaweed alginate finds numerous industrial and medical applications, alginate produced by *Azotobacter* and *Pseudomonas* spp., is important for cyst and biofim formation, respectively. A member of *Pseudomonas, Pseudomonas aeruginosa*, is the leading cause of death in Cystic Fibrosis (CF) patients. This pathogen over-produces alginate upon infection of the CF lung, protecting it from host immune responses and antibiotics while clogging up the patients' airways leading to poor prognosis. Alginate biosynthesis occurs in four stages: (1) precursor synthesis in the cytoplasm (AlgA, D and C), (2) polymerisation at the inner membrane (Alg8 and Alg44), (3) periplasmic translocation and modification (AlgK, X, L, G, I, J and F), and (4) secretion (AlgE) across the outer membrane. The latter three stages are facilitated by a putative multi-protein complex spanning the entire envelope fraction. Currently, it is unknown how this complex is assembled and the roles certain components of the complex play in alginate biosynthesis are not clear. The periplasmic protein AlgK is a key component of this complex. This protein has multiple protein-protein interaction domains, suggesting that it could be critical for assembling functional alginate biosynthesis machinery. In the present study, an *algK* mutant was generated and used to determine the impact of AlgK's absence on (i) alginate yield and size, and (ii) the stability of other components of the alginate biosynthesis machinery. This study demonstrates that AlgK is essential for polymerisation and is required for the stability of components involved in polymerisation (Alg44), translocation (AlgX), and secretion (AlgE). We also show that AlgK interacts with periplasmic AlgX but not with inner membrane Alg44 or outer membrane AlgE. Overall, this study sheds light on the role of AlgK in alginate production and the assembly of the alginate biosynthesis machinery.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor Bernd Rehm, for this opportunity to study in his laboratory. It has been a privilege to work under his supervision. He has been a great source of wisdom and encouragement and always willingly provided advice and help with a smile. I would also like to thank the Rehm Laboratory team for being such a pleasant and productive group of people to work with. My gratitude also goes to the former Institute of Molecular Biosciences for providing excellent facilities and a friendly working environment.

I am grateful to Dr. Zahid Rehman for training me in the art of handling and analysing DNA, alginate and proteins. I would also like to acknowledge his assistance in completing the mutual stability experiments and for generating the *in cis* complemented PDO300Δ*algE*, Δ*alg44* and Δ*algX* mutant strains. I am thankful to Dr. Iain Hay for his valuable advice and critique of my thesis and Mr. M. Fata Moradali for his enthusiasm and helping me optimise pull down experiments. I would also like to recognise the efforts of the Rehm Laboratory manager, Ms. Natalie Burr, for making everything go smoothly.

I would like to thank Professor Barry Scott and Mr. Chris Burrows for kindly allowing me to use their ultra-centrifuges, Dr. Mark Patchett for providing electroporation equipment, Dr. Gill Norris for her insightful guidance on protein-protein interactions and providing the French Press for cell disruption, Mr. Robert Cleaver for training me to use the ultra-centrifuges, Mr. Trevor Loo for training me on the French Press, Associate Professor Rosie Bradshaw for sparing me chemicals and allowing me to borrow her spectrophotometer, Dr. Jasna Rakonjac for storing my samples in her freezer when our minus eighty was out of order, Professor Michael McManus for providing me with liquid nitrogen, and Associate Professor Kathryn Stowell for the Nano Drop spectrophotometer and for being a supportive postgraduate coordinator.

I would also like to extend my gratitude to the Massey Genome Service for sequencing my plasmids, Mrs. Natalie Gardner and her wonderful kitchen colleagues for autoclaving my media, glassware, and disposal of experimental waste, the Allan Wilson Centre for providing freeze-drying equipment, Ms. Katrina Ross, Mr. Paul Hocquard

and Ms. Tara Penketh for making the money and ordering side of things go super smooth, and Mrs. Ann Truter and Mrs. Cynthia Creswell for making all administrative matters a breeze.

I am grateful to the paper co-ordinators for my postgraduate papers, and Dr Zoe Jordens and everyone that taught me throughout my under and postgraduate studies at Massey University. You have reinforced my fascination on how and why biological things work at the molecular level.

I also thank my friends Tian Yang, Mia Lu and Kelly Zhu for the fun times we had.

Finally, I would like to thank my family for their unconditional love and support. My special gratitude goes to my father for his encouragement, my late mother for being proud of my achievements, my stepmother for supporting my father and cooking wonderful food that I get to eat in front of my envious peers, and my little brother for hugging me when my experiments failed.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A full list of abbreviations used

°C	Degree Celsius
AGE	Agarose gel electrophoresis
Ap	Ampicillin
APS	Ammonium persulfate
BSA	Bovine serum albumin
Cb	Carbenicillin
Δ	Delta (deleted)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
EtOH	Ethanol
EDTA	Ethylenediaminetetraacetic acid
g	gravity/gram
Gm	gentamycin
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic
acid	
HRP	Horse radish peroxidase
IPTG	Isopropyl â-D-1-thiogalactopyranoside
kDa	Kilo Daltons
λ	Lambda (wavelength or type of phage)
LB	Luria-Bertani (broth)
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar
PPI	Protein-protein interaction
RNAase	Ribonuclease
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate gel electrophoresis

TBE	Tris-Borate-EDTA buffer
Tc	Tetracycline
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
T_m	Primer melting temperature
Tris	Trishydroxymethylaminomethane
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Alginate is a collective term for a family of polysaccharides produced by brown seaweeds and bacteria (Rehm 2009). In brown seaweeds, alginate plays an essential role in maintaining the structure of algal tissues. Alginate harvested from brown seaweeds finds numerous industrial, pharmaceutical and medical applications (Qin 2008). Alginate also plays an essential role in bacteria. In *Azotobacter* spp., it is required for formation of desiccation resistant cysts while in *Pseudomonas* spp., it is essential for biofilm development (Hay et al. 2010a). A member of the latter genus, *Pseudomonas aeruginosa*, is the leading cause of death in Cystic Fibrosis (CF) patients (Hay et al. 2010a). Upon infection, *P. aeruginosa* switches to a mucoid phenotype characterised by the over-production of alginate which clogs up the patients' lungs and protects the pathogen against antibiotics and host immune responses. Decades' research has unravelled the identity, structure and function of proteins involved in alginate biosynthesis and regulation. In this chapter I first described the structure and applications of alginate (Section 1.1), and the relationship between *P. aeruginosa* and CF (Section 1.2). I then reviewed the genetics (Section 1.3) and regulation (Section 1.4) of alginate production by *P. aeruginosa.* In Section 1.5, I described the biosynthesis of alginate by this organism. Finally, I introduced the focus of my study, AlgK, a protein involved in alginate production by *P. aeruginosa* (Section 1.6) as well as the aims and objectives (Section 1.7) and hypotheses of this study (Section 1.8).

1.1 Alginate structure and applications

Alginate is a linear unbranched non-repeating anionic copolymer composed of varying quantities of beta-D-mannuronic acid (M) and its c-5 epimer, alpha-L-guluronic acid (G), which are linked via 1,4 glycosidic bonds (Figure 1). These monomers form continuous mannuronic acid (MM), guluronic acid (GG), and alternating residues (MG) (Rehm 2010).

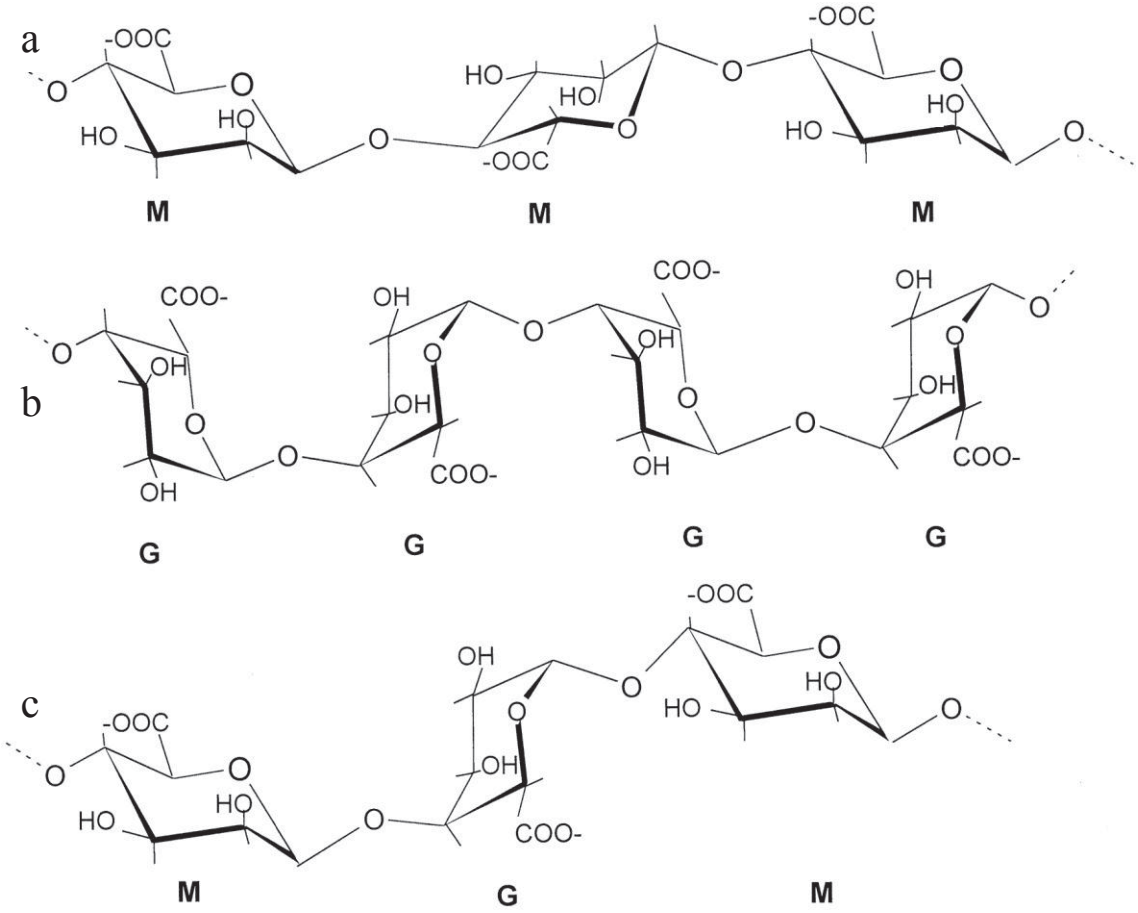

Figure 1. Chemical structure of alginate. Alginate is a linear anionic copolymer of 1-4 linked β-D-mannuronic acid (M) and its C_5 epimer, α-L-guluronic acid (G). These monomers form homopolymeric blocks of poly-mannuronate (MM) (a) and poly-guluronate (GG) (b), and heteropolymeric blocks with random arrangements of both monomers (MG) (c) (Rehm 2010).

Alginate was first isolated from brown seaweeds during the late 19th century. Since then, it has become an important commercial product with over 30,000 metric tons harvested annually (Draget et al. 2005). Owing to its versatility and biocompatibility, alginate is used as a viscosity regulator and stabiliser in foods, cosmetics and high value medical applications including wound dressings, drug delivery systems, and more recently in tissue encapsulation for regenerative therapy (Figure 2, Tonnesen & Karlsen 2002, Selden & Hodgson 2004, Qin 2008, Lim et al. 2010). In the wound management industry alginate in its unmodified form isolated from seaweeds is extensively used due to its non-immunogenicity and excellent ion-exchange and gel-forming properties (Qin

2008). Alginate wound dressings can absorb large volumes of exudates while maintaining a moist environment to promote wound healing (Thomas et al. 2000). It has also been shown that alginate based wound dressings augmented with anti-microbial agents such as zinc and silver ions can potentially reduce healing time (Agren 1999, Wilkinson et al. 2011).

Figure 2. Applications of alginate. Alginate is used as a viscosity regulator, stabiliser and gelling agent (from top left) in ice cream, beer and pharmaceuticals, as well as (from bottom left) bandages and cosmetics. All images are used with permission from Microsoft.

The demand for alginate in drug delivery systems and tissue encapsulation technologies has pushed for the development of tailor-made polymers. Alginate can be tailored by enzymatic and chemical means to meet the requirements of these high value applications (Goh et al. 2012). The structure and physicochemical properties of alginate can be modified by naturally occurring enzymes such as epimerases, acetyltransferases,

and lyases (Rehm 2010). For instance, *Azotobacter* produces eight epimerases essential for cyst formation, several of which are used to generate alginate with improved gel forming and pH dependent properties (Bjerkan et al. 2004, Hartmann et al. 2006). Alginate can also be modified by chemical means such as acetylation, phosphorylation, sulfation and addition of hydrophobic groups (Alban et al. 2002, Coleman et al. 2011). Acetylation allows formation of low strength gels with increased water binding capacity while phosphorylation increases gel strength – a modification particularly useful for hydroxyapatite nucleation and growth with potential applications in chromatography, drug delivery and bone replacement (Coleman et al. 2011). In contrast, addition of sulphate groups introduces blood-compatibility and anticoagulant activity while offering better control over the release of growth factors. Hydrophobic modifications can also provide controlled release of polypeptide drugs (Alban et al. 2002).

Alginate has great potential in tissue microencapsulation technology whereby cells are immobilising within a semi-permeable alginate matrix, allowing free diffusion of nutrients and waste while protecting cells from immune responses (Goh et al. 2012). Other desirable features of modified alginate include temperature and pH dependent properties as well as surfaces designed for cell attachment (Jao et al. 2009, Steward et al. 2011). Modified alginate is often combined with other polymeric substances to meet industry requirements (Steward et al. 2011).

However, alginate isolated from brown seaweeds – our current source of this polymer - is subject to heterogeneity due to seasonal variation; this heterogeneity often limits its use (Qin 2008). Bacterial alginate, on the other hand, is more homogenous but is currently too expensive to produce commercially (Rehm 2010). Understanding and harnessing the mechanisms of alginate production in bacteria could enable manufacture of designer bacteria-derived alginate for high value medical applications.

1.2 *Pseudomonas aeruginosa* and Cystic Fibrosis

Cystic Fibrosis (CF) is a hereditary disease (Figure 3) which claims 30,000 lives annually among the Caucasian population (O'Sullivan & Freedman 2009). These patients have a faulty gene called CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) which produces a defective cAMP-dependent chloride channel that drastically diminishes the control of salt and water transport by cells (Stutts et al. 1995). Over 1,900 mutations of this gene have been catalogued in the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/Home.html). The defective channel leads to a thickening of epithelial secretions that obstruct various organs including the pancreatic duct, intestinal glands and bronchi (Figure 3, O'Sullivan & Freedman 2009). These obstructions prevent proper secretion of digestive enzymes and removal of foreign matter from lungs, leading to malnutrition and chronic lung infection (Rowe et al. 2005). Malnutrition can generally be treated by dietary adjustments and administration of deficient enzymes. However, chronic lung infection is a lot more difficult to manage. This is reflected by the shocking statistics: approximately 80% of CF fatalities in America are due to pulmonary insufficiency associated with chronic lung infection (Kumar et al. 2007).

Chronic lung infection leads to chronic inflammation, cystic bronchiectasis and severe airflow obstruction leading to patient mortality in their mid-thirties (Rowe et al. 2005, Williams et al. 2010). *P. aeruginosa* infection is the leading cause of morbidity and mortality in CF patients. This ubiquitous gram negative bacterium is also a common pathogen of other mammals, protozoa and plants. Owing to its many nutrient acquisition pathways and defence mechanisms this it can survive environmental extremes, starvation and bombardment of antimicrobial agents (Williams et al. 2010). These characteristics along with its large plastic genome and ability to grow as a biofilm and over-produce alginate provide it with distinct advantages (Stover et al. 2000). For instance, its genome plasticity allows rapid accumulation of mutations for adaptation to a new environment (Mathee et al. 2008). This is particularly pronounced during biofilm growth where the mutation rate is significantly enhanced by oxidant-induced double-strand DNA breaks and deficient repair mechanisms (Boles et al. 2005, Boles & Singh 2008, Mena et al. 2008). Although *P. aeruginosa* infections are generally acquired from the environment, the strains isolated from the lungs of CF patients are better adapted for

establishing chronic lung infection than their environmental counterparts (Bragonzi et al. 2009).

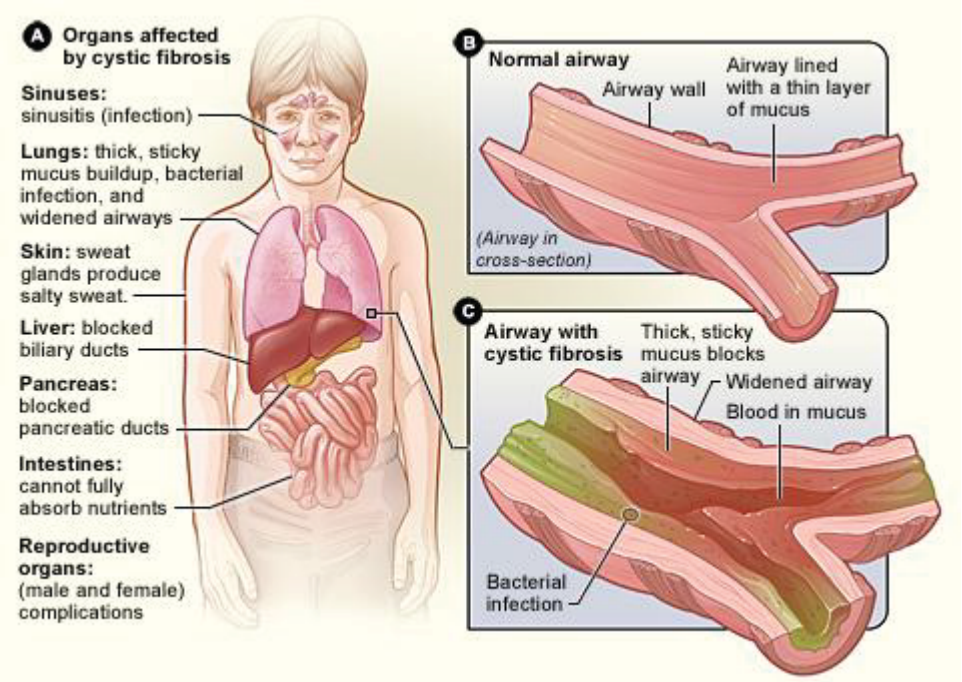

Figure 3. Cystic Fibrosis. Cystic Fibrosis (CF) patients have a mutated CFTR gene that produces a defective cAMP-dependent chloride channel which impairs control of salt and water secretion of cells. This leads to an accumulation of thick secretions that impair the function of numerous organs including the lungs (A). The cross-section of normal (B) and CF (C) lung airways shows that the accumulation of mucus in the CF lung leaves patients susceptible to bacterial infection. Chronic pulmonary infection by *P. aeruginosa* is the leading cause of death in CF patients. "Cystic fibrosis signs & symptoms." *NIH Heart, Lung and Blood Institute*. Web. 12 Feb. 2013. <http://www.nhlbi.nih.gov/health//dci/Diseases/cf/cf_signs.html>.

P. aeruginosa establishes in the thick airway mucus of the CF lung, rarely coming into contact with cell surfaces (Moreau-Marquis et al. 2008). Cellular debris and particulates trapped in the thick mucus provide adequate surface area for colonisation (Whitchurch et al. 2002). Upon infection, *P. aeruginosa* transforms into a state of persistence rather than destruction to evade host detection and defences (Smith et al. 2006). Host detection is avoided by down-regulating surface and secreted virulence traits while the over-production of alginate provides protection against host defences such as phagocytosis and reactive oxygen species (Coban et al. 2009, Harmsen et al. 2010). This polymer

also blocks antibiotic penetration, making mature alginate-rich biofilms incredibly difficult to eradicate (Pedersen et al. 1990). Current treatment strategies require early detection and aggressive use of antibiotics. However, alternative therapies for treatment have also emerged, for instance, supplementing antibiotics with alginate degrading enzymes (Mrsny et al. 1996). Recently, bacteriophages producing alginate lysases have also been successfully used to clear *P. aeruginosa* infection in a murine model (Alemayehu et al. 2012).

1.3 Genetics of alginate production

At least 24 genes are directly involved in alginate production by *P. aeruginosa* (Table 1) (Hay et al. 2010a). The structural biosynthesis genes are located on two operons: the *algD* operon containing the *algD, 8, 44, K, E, G, X, L, I, J, F & A* genes, and the *algC* operon containing the *algC* gene (Muhammadi & Ahmed 2007). The gene products of *algD*, *algA*, and *algC* are involved in precursor synthesis; *alg8* and *alg44* in polymerisation; *algG* in epimerisation; *algI, J, F* in acetylation; *algL* in alginate degradation; *algX* and *algK* in alginate translocation across the periplasm, and *algE* in secretion (Hay et al. 2010a). Both operons are under strict control by their respective upstream promoters (Rehm & Valla 1997). Although the arrangement of genes (*algD* to *algA*) suggests that they form an operon, the full length polycistronic transcript has never been isolated. There is evidence for weak promoters, other than the *algD* promoter, directly upstream of *algE*, *algG* and *algI* (Chu et al. 1991, Paletta & Ohman 2012).

Numerous genes regulate alginate production by *P. aeruginosa*. The *algU* operon, the switch of alginate biosynthesis, contains the genes *algU, mucA, mucB, mucC* and *mucD* which encode an alternative sigma factor (AlgU), its negative regulators (MucA, MucB and MucD), and a protein with an unclear role (MucC) (Mathee et al. 1997, Cezairliyan & Sauer 2009, Wood & Ohman 2009). Transcription of the *algD* and *algC* operons is also regulated by several other genes encoding an anti-sigma factor (*algQ*), two component sensory transduction systems (*algZ/algR* and *kinB/algB*), DNA binding proteins (*amrZ*), and histone-like proteins (*algP*) (Kato et al. 1990, Ma et al. 1998, Wozniak et al. 2003, Wood & Ohman 2009, Yuan et al. 2008). In addition, alginate production is regulated post-transcriptionally and post-translationally by the genes

MucD-AS and *mucR* which encode a natural anti-sense transcript and an alginate specific c-di-GMP synthase (Hay et al. 2009, Yang et al. 2011).

TABLE 1. Genes and their roles in alginate production (Hay et al. 2010a).

Gene	Function
algA	Phosphomannose isomerase/GDP mannose pyrophosphorylase
algC	Phosphomannomutase
algD	GDP mannose dehydrogenase
alg8	Glycosyltransferase/polymerase function
alg44	c-di-GMP binding regulation/membrane fusion
algI	O-Acetylation
algJ	O-Acetylation
algF	O-Acetylation
algG	Mannuronan C-5 epimerase
algL	Alginate lyase
algK	Periplasmic scaffold/scaffold assembly
algX	Periplasmic scaffold/sequesters MucD
algE	Outer membrane alginate secretion porin
mucR	Alginate specific diguanylate cyclase (c-di-GMP synthase)
algU	Global stress response factor (σ 22)
mucA	Anti σ 22 factor
mucB	Anti σ 22 factor
mucC	Unknown role
mucD	Homologue of *E. coli* serine protease DegP
mucE	Misfolding protein activating RIP cascade to release AlgU
algW	Protease releasing AlgU via RIP cascade
mucP	Protease releasing AlgU via RIP cascade
algQ	Anti σ 70 factor
algR	Regulatory component of TCST system
algZ	AlgR cognate sensor kinase
algB	Regulatory component of TCST system
kinB	AlgB cognate senor kinase
amrZ	DNA binding protein
algP	Histone-like transcription regulator

1.4 Regulation of alginate biosynthesis

Alginate biosynthesis by *P. aeruginosa* is controlled by a complex regulatory network responding to changes in the intracellular and extracellular environment.

1.4.1 Phenotypic switching

The *algU* operon (*algU, mucA, mucB, mucC* and *mucD*), a hyper-mutable region (Figure 4), is the switch for alginate biosynthesis where mutations especially in *mucA* cause excessive alginate production and a switch to the mucoid phenotype (Mathee et al. 1997). *algU* encodes an alternative sigma factor, AlgU, which activates transcription of several operons involved in alginate regulation and biosynthesis: *algU, algR*, *algB*, *algD*, *algC* and *amrZ* (Firoved et al. 2002, Wozniak et al. 2003, Muhammadi & Ahmed 2007). It is normally sequestered at the cytoplasmic membrane by its anti-sigma factors, the inner membrane proteins MucA and MucB (Cezairliyan & Sauer 2009, Wood & Ohman 2009). Under conditions of envelope stress AlgU is released from its anti-sigma factor complex by a regulated intramembrane proteolysis (RIP) cascade involving the periplasmic AlgW and Prc, inner membrane MucP, and cytoplasmic ClpXP proteases (Figure 4, Wood et al. 2006, Qiu et al. 2007, Qiu et al. 2008b, Cezairliyan & Sauer 2009). Envelope stress induces misfolding of proteins, one of which, MucE, in its misfolded form activates the proteolytic activity of AlgW through its C terminal WVF triplet motif (Qiu et al. 2007, Wood & Ohman 2009). AlgW could also potentially be activated by other envelope proteins carrying the WVF or similar motifs (Qiu et al. 2008a, Damron & Goldberg 2012).

Within the *algU* operon there are two other genes, *mucC* and *mucD* encoding MucC and MucD, respectively (Boucher et al. 1997, Wood & Ohman 2006). Although the role of MucC is unclear, a promoter region in the *mucC* ORF is essential for the expression of its downstream gene, *mucD* (Wood & Ohman 2006). The gene product of *mucD* (MucD) is a serine protease and chaperone-like protein that negatively regulates alginate biosynthesis through degradation of misfolded MucE (Qiu et al. 2007, Cezairliyan & Sauer 2009). The liberation of AlgU from MucA-MucB in *P. aeruginosa* is homologous to the release of the alternative sigma factor, RpoE, from its cognate anti-sigma factor complex, RseA-RseB, in *Escherichia coli* (Rowley et al. 2006, Wood

et al. 2006). The proteases controlling AlgU and RpoE liberation have PDZ domains likely to be involved in protein-protein interactions (Rowley et al. 2006). PDZ is an acronym combining the first three letters of three proteins - post synaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) (Kennedy 1995). The PDZ domain of AlgW is required for signal transduction and possibly for trimerisation (Qiu et al. 2006). The two PDZ domains of MucD may also be involved in substrate binding. Recently, MucD was shown to interact with components of the alginate biosynthesis machinery, AlgX and AlgK (Gutsche et al. 2006, Hay et al. 2012).

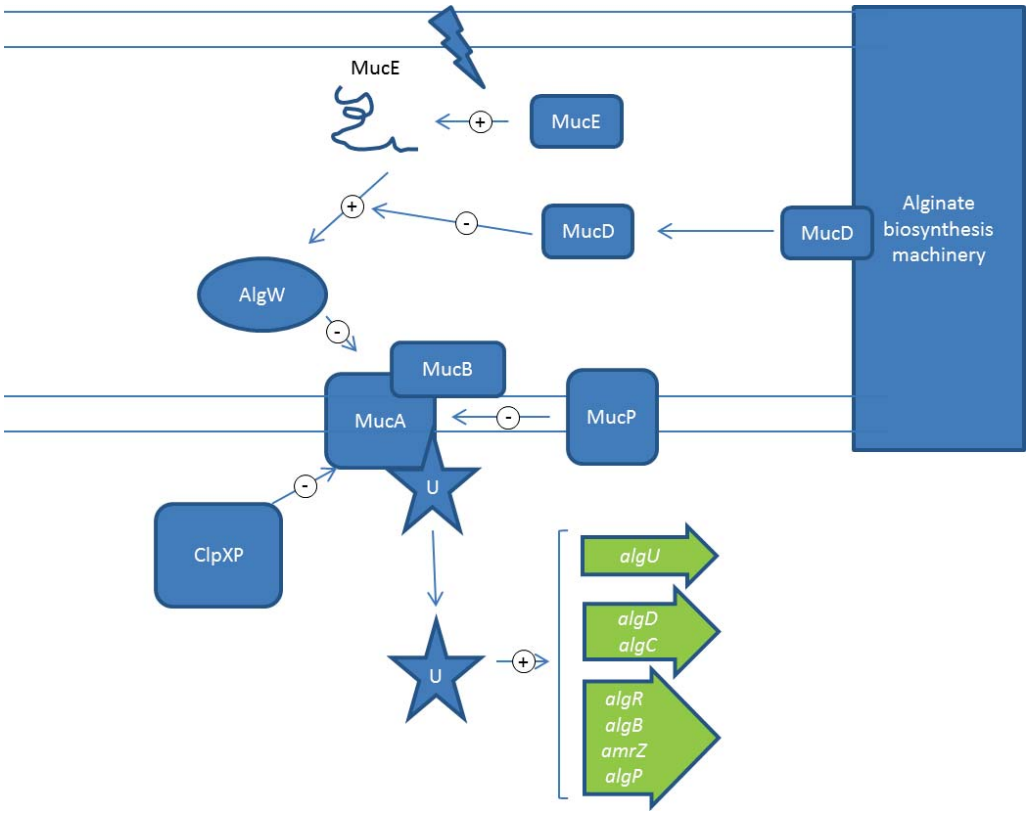

Figure 4. Phenotypic switching. Under conditions of envelope stress (lightning bolt), MucE becomes mis-folded ('MucE' squiggle) and initiates a regulated intra-membrane proteolysis (RIP) cascade, releasing AlgU from its anti-sigma factor complex (MucA-MucB) (Rowley et al. 2006). The liberation of AlgU involves several proteases, AlgW, MucP and ClpXP, which sequentially degrade MucA and MucB (Wood et al. 2006, Qiu et al. 2007, Qiu et al. 2008b, Cezairliyan & Sauer 2009). The liberated AlgU initiates gene expression from several operons involved in alginate production (green block arrows). MucD is a protease that degrades misfolded MucE which would otherwise activate the RIP cascade. The arrows and +/- indicate direct initiation or inhibition of downstream genes or proteins.

1.4.2 Transcriptional regulation

Alginate production is regulated transcriptionally by several proteins (Figure 5). AlgQ is the cognate anti-sigma factor of the default sigma factor σ^{70}. It promotes alginate production by preventing σ^{70} from interacting properly with RNA polymerase, allowing alternative sigma factors such as AlgU to take control of transcription (Pineda et al. 2004, Yuan et al. 2008). Alginate production is also transcriptionally regulated by DNA binding proteins such as AmrZ and AlgP which promote *algD* operon expression by latching onto its promoter (Kato et al. 1990, Baynham et al. 2006). The versatility of AmrZ's DNA binding domain allows it to repress and activate expression of genes at alternative promoters, for instance, AmrZ represses the expression of its gene while activates transcription of the *algD* operon (Wozniak et al. 2003, Ramsey et al. 2005).

The expression of alginate production genes is also regulated by the cognate sensor/regulator elements of two component signal transduction systems such as AlgZ/AlgR and KinB/AlgB (Ma et al. 1998). Typically, the cognate sensor upon stimulation by an environmental cue transfers a phosphate group to the regulating element, activating its regulating properties. The response regulator, AlgR, binds to the *algD* promoter at three locations, activating the transcription of this operon (Ma et al. 1998). Intriguingly, its cognate sensor regulator, AlgZ, is not required for its regulation of alginate production (Ma et al. 1998). AlgB is another response regulator which activates the expression of the *algD* operon; however, it too does so independent of its cognate sensor regulator, KinB (Wozniak & Ohman 1993, Ma et al. 1998).

Because so many DNA binding proteins regulate *algD* operon expression, it is apparent that the three-dimensional nucleoid structure of the *algD* promoter greatly impacts transcription efficiency. Thus, different combinations of regulatory elements binding *algD* and other alginate related promoters would affect gene expression and the quantity of alginate produced. It is important to note that the transcriptional regulators which control expression of alginate genes also regulate numerous other pathways including quorum sensing, biofilm formation and motility, metabolite synthesis and nutrient acquisition, and various virulence factors important for lung infection (Lizewski et al. 2004, Baynham et al. 2006, Yuan et al. 2008, Damron et al. 2012).

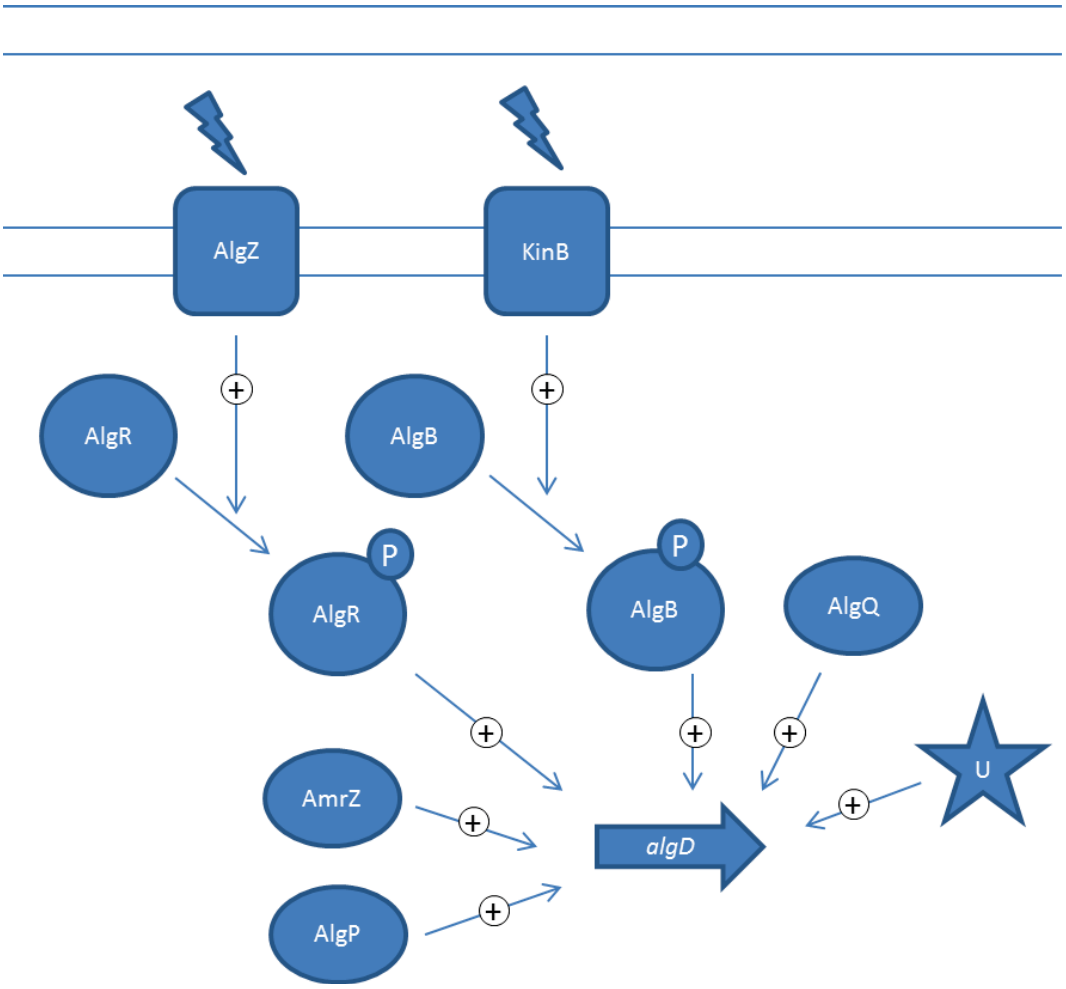

Figure 5. Transcriptional regulation of alginate biosynthesis. Expression of the *algD* operon (blue solid arrow) is positively regulated by the alternative sigma factor AlgU, the anti-sigma factor AlgQ, DNA binding proteins AmrZ and AlgP, and sensor/regulatory elements of two component signal transduction systems (AlgZ/AlgR and KinB/AlgB). AlgQ promotes alginate production by preventing σ^{70} from interacting properly with RNA polymerase, allowing alternative sigma factors such as AlgU to take control of transcription while AmrZ and AlgP promote alginate production by binding to and initiating gene expression from the *algD* promoter (Kato et al. 1990, Baynham et al. 1996, Pineda et al. 2004, Yuan et al. 2008). The cognate sensor elements AlgZ and KinB under stimulation by an unidentified cue (lightning bolts) phosphorylate their cognate response elements (by addition of "P" groups to AlgR and AlgB, respectively) which bind to and activate transcription from the *algD* promoter. Intriguingly, the sensor elements AlgZ and KinB are not essential for AlgR and AlgB mediated transcription of the *algD* operon (Ma et al. 1998). The arrows and +/- indicate direct initiation or inhibition of downstream genes or proteins.

1.4.3 Post-translational regulation

Alginate biosynthesis is regulated post-translationally by bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) binding to the PilZ domain of Alg44, the putative co-polymerase of the alginate biosynthesis machinery (Figure 6, Merighi et al. 2007, Hay et al. 2009). c-di-GMP is a generic secondary messenger molecule utilised by bacteria for regulation of multiple systems involved in motility, exopolysaccharide production and virulence (Romling et al. 2005, Hay et al. 2010a). A highly specific protein, MucR, is thought to synthesise a localised pool of c-di-GMP in close proximity to Alg44 upon undetermined environmental stimulation (Hay et al. 2009). Interestingly, MucR also has a c-di-GMP degrading domain that is required for alginate biosynthesis (Hay et al. 2009).

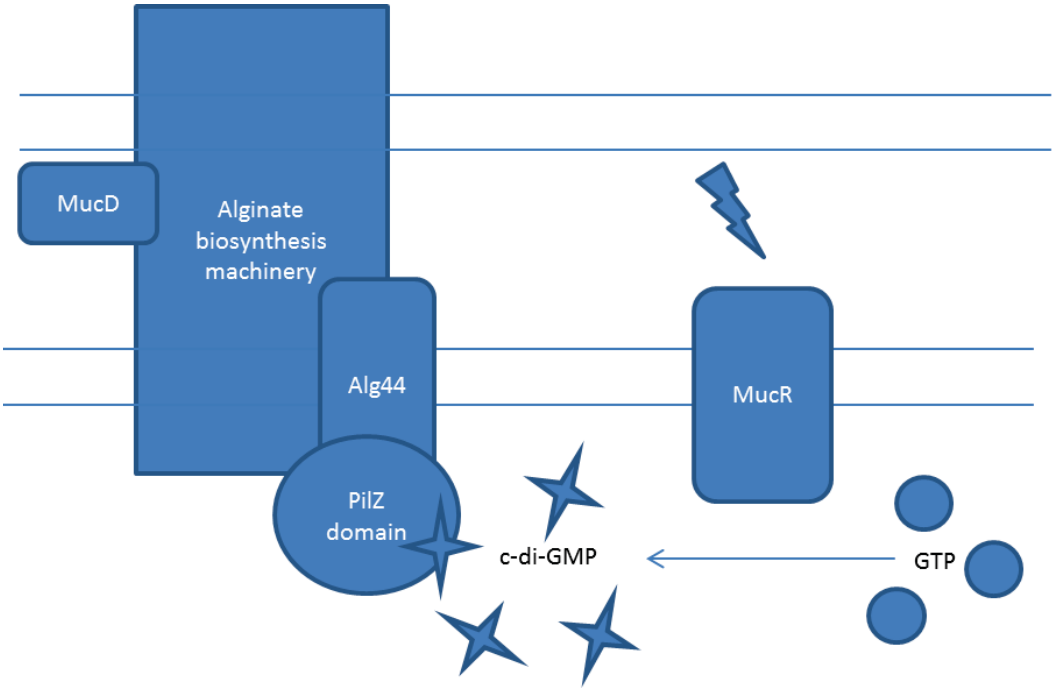

Figure 6. Post-translational regulation of alginate biosynthesis. Upon stimulation by an unidentified cue (lightning bolt), MucR synthesises c-di-GMP (four sided stars) from GTP (circles) which binds to the PilZ domain of Alg44, the co-polymerase facilitating alginate biosynthesis. Both MucR and the PilZ domain of Alg44 are required for alginate production. However, it is unclear whether the binding of c-di-GMP facilitates the assembly of the biosynthesis machinery or induces conformational changes necessary for polymerisation/secretion.

1.5 Biosynthesis of alginate

The chemical pathway for alginate production by *P. aeruginosa* is presented in Figure 7. At the start of alginate biosynthesis the alginate precursor, GDP mannuronic acid, is synthesised in the cytoplasm from the central metabolite fructose-6-phosphate (Section 1.5.1). The precursor is then polymerised and translocated across the cytoplasmic membrane (Section 1.5.2). Thereafter, the alginate chain is translocated across the periplasm where it is modified by several enzymes (Section 1.5.3). Finally, the mature alginate is secreted across the outer membrane (Section 1.5.4).

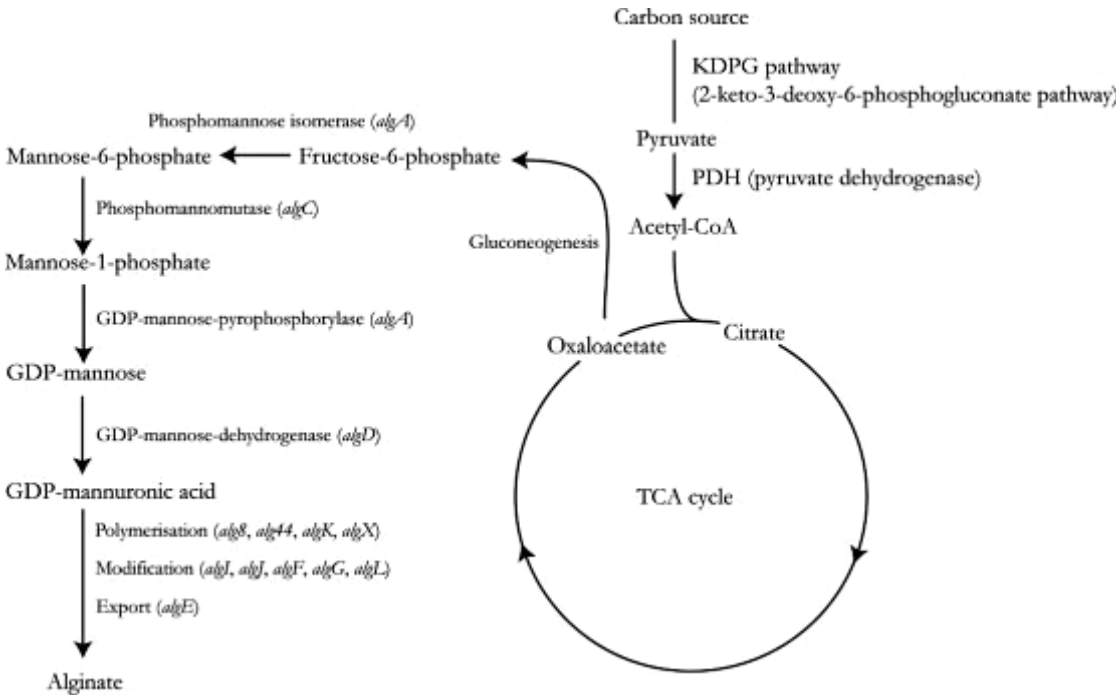

Figure 7. Alginate biosynthesis pathway. The alginate biosynthesis pathway is presented by Hay et al. (2010a) in a recent literature review. A carbon source is converted to pyruvate through the KDPG pathway. Pyrvuate is converted to acetyl-CoA, which enters the TCA cycle. Oxaloacetate from the TCA cycle is turned into fructose-6-phosphate through gluconeogenesis. Fructose-6-phosphate is transformed into GDP-mannuronic acid, the alginate precursor, through a series of reactions catalysed by AlgA, AlgC and AlgD. This precursor is polymerised into alginate, which is modified and then secreted out of the cell by a transmembrane multi-protein complex consisting of ten proteins Alg8, Alg44, AlgK, AlgX, AlgI, AlgJ, AlgF, AlgG, AlgL and AlgE.

A putative multi-protein complex spanning the inner and outer membranes and periplasm facilitates the polymerisation, modification, translocation and secretion of alginate (Figure 8). The current model proposes that when subunits required for polymerisation are missing, alginate is not produced. Conversely, when subunits required for translocation or secretion are missing, alginate is still being polymerised but is degraded in the periplasm, releasing alginate degradation products in the form of free uronic acid oligomers.

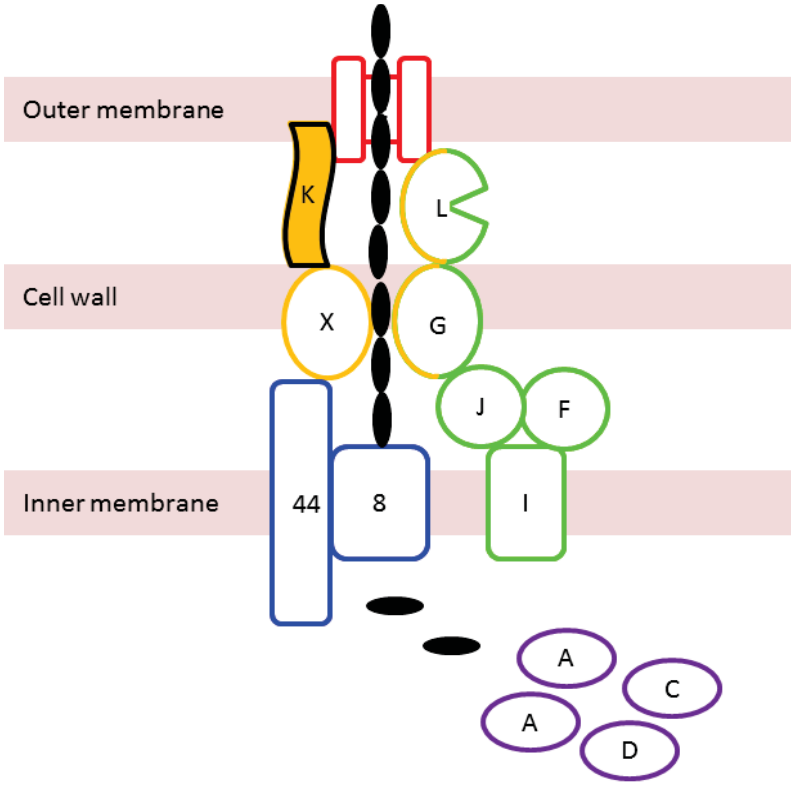

Figure 8. Alginate biosynthesis by *P. aeruginosa*. Thirteen proteins facilitate alginate biosynthesis. The three cytoplasmic proteins AlgA, AlgD and AlgC (purple ovals) synthesise the precursor, GDP-mannuronic acid (solid black ovals), which is polymerised by the inner membrane proteins Alg8 and Alg44 (blue rectangles) forming the nascent alginate chain. This is translocated across the periplasm by a multi-protein scaffold (AlgG, AlgX, AlgL and AlgK) to AlgE (red protein) for secretion. The nascent alginate chain is epimerised by AlgG, acetylated by AlgI, J and F, and terminated by AlgL in the periplasm. Proteins are colour-coded according to their proposed role: purple for precursor synthesis, blue for polymerisation and cytoplasmic membrane transfer, green for modification, orange for periplasmic translocation, and red for secretion across outer membrane. Here, AlgK, the focus of this study is highlighted (black outline with solid orange centre). The position of the cell wall is only for illustrative purposes. Similarly, the contact between proteins only represents conceivable interactions.

1.5.1 Precursor synthesis in cytoplasm

The alginate precursor, GDP-mannuronic acid, is synthesised from the central metabolite fructose-6-phosphate by AlgA, AlgC and AlgD (Figure 7 & 8). Fructose-6-phosphate is converted into mannose-6-phosphate by the dual functional protein AlgA through its phosphomannose isomerase activity (May et al. 1994). Then, AlgC turns mannose-6-phosphate into mannose-1-phosphate (Zielinski et al. 1991). In the third reaction, mannose-1-phosphate is converted into GDP-mannose by the GDP-mannose pyrophorylase activity of AlgA (Shinabarger et al. 1991). Finally, AlgD converts GDP-mannose into GDP-mannuronic acid, the precursor of alginate biosynthesis (Tatnell et al. 1994). Although this reaction is considered the first committed step in alginate biosynthesis because it is irreversible, it is not considered a rate limiting step because over-expression of *algD* only marginally increases alginate yield, suggesting that some other reaction is the bottleneck (Roychoudhury et al. 1989, Tatnell et al. 1994).

1.5.2 Polymerisation and translocation across cytoplasmic membrane

The alginate precursor, GDP-mannuronic acid, is polymerised into poly-mannuronate by two inner membrane proteins, Alg8 and Alg44 (Figure 8, Remminghorst & Rehm 2006a, Remminghorst & Rehm 2006b). Neither *alg8* nor *alg44* mutants could synthesise alginate or its degradation products (Maharaj et al. 1993, Remminghorst & Rehm 2006a, Remminghorst & Rehm 2006b, Oglesby et al. 2008). Alg8 has homologies with class II beta glycosyltransferases that add a monomer sugar or sugar derivative to the end of a substrate such as a polysaccharide chain (Remminghorst et al. 2009). Over-expression of either Alg8 or Alg44 has been found to significantly increase alginate production, suggesting that the polymerisation step is a key bottleneck (Remminghorst & Rehm 2006a, Remminghorst & Rehm 2006b). Alg8 has several conserved sugar binding DXD motifs required for alginate polymerisation (Remminghorst et al. 2009, Oglesby et al. 2008). These motifs are present in numerous glycosyltransferases, which add a range of different sugars to other sugars or proteins and typically use nucleoside diphosphate sugars as donors (Wiggins & Munro 1998).

Alg44 is considered a co-polymerase playing potential catalytic, regulatory and structural roles (Remminghorst & Rehm 2006a, Merighi et al. 2007). It has a trans-membrane domain separating a cytoplasmic PilZ and a periplasmic membrane fusion domain (MFP); both of these domains are required for alginate production (Remminghorst & Rehm 2006a, Merighi et al. 2007). The PilZ domain participates in post-translational regulation; it binds the secondary messenger molecule c-di-GMP which is produced by MucR, a regulator of alginate biosynthesis (Figure 6, Amikam & Galperin 2006, Merighi et al. 2007, Hay et al. 2009). The binding of c-di-GMP to this domain could elicit an essential conformational change, allowing the assembly or activation of the alginate biosynthesis machinery. By comparison, the MFP domain of Alg44 could play a structural role by bridging the cytoplasmic subunits of the alginate biosynthesis machinery with periplasmic and/or outer membrane components. This domain shares homology to periplasmic adaptor proteins of the tripartite multidrug efflux pumps such as MexA which has been shown to interact with the inner membrane transporter MexB and the outer membrane exit duct OprM (Merighi et al. 2007, Lobedanz et al. 2007, Symmons et al. 2009). Similarly, Alg44 has been shown to be required for the stability of AlgE, the outer membrane protein facilitating alginate secretion (Oglesby et al. 2008).

1.5.3 Translocation across periplasm and modification

After polymerisation, the nascent alginate chain, poly-mannuronate, is translocated across the periplasm by a putative multi-protein scaffold consisting of the periplasmic proteins AlgX, AlgG and AlgK (Figure 8 & 10, Jain & Ohman 1998, Jain et al. 2003, Robles-Price et al. 2004). These proteins are thought to guide the nascent alginate chain through the periplasm while protecting it from degradation by the alginate lyase, AlgL (Jain & Ohman 1998, Jain et al. 2003, Robles-Price et al. 2004). Some authors have proposed that AlgL also contributes to the periplasmic translocation of alginate (Jain & Ohman 2005). When components of the periplasmic scaffold are missing, the nascent alginate chain leaks into the periplasm and is degraded by AlgL, releasing free uronic acid oligomers (Jain & Ohman 1998, Jain et al. 2003, Robles-Price et al. 2004).

Although several studies have indicated that AlgX is essential for alginate production, its exact role is unclear (Monday & Schiller 1996, Robles-Price et al. 2004, Gutsche et

al. 2006). Robles-Price et al. (2004) have proposed that AlgX forms part of the periplasmic scaffold facilitating alginate translocation and secretion. In contrast, studies by Gutsche et al. (2006) supported the possibility that AlgX was required for efficient polymerisation. Both AlgX and AlgG have sugar-binding-hydrolysing domains likely to be involved in substrate binding (Robles-Price et al. 2004, Douthit et al. 2005). AlgG also has a C-5 mannuronan epimerase activity, which converts M residues to G residues. AlgK, on the other hand, has multiple tetratricopeptide-like (TPR-like) repeats, a feature characteristic of proteins involved in the assembly of multi-protein complexes, suggesting that AlgK may play an important role in the assembly of functional alginate biosynthesis machinery (Keiski et al. 2010). Recently, AlgK has been shown to interact with AlgX and MucD – the latter is a regulatory protein, suggesting that AlgK may also have regulatory roles in alginate production (Hay et al. 2012).

While the nascent alginate chain is transported across the periplasmic space, it can be modified by o-acetylation, epimerisation and degradation. Although the order of modification remains unknown, it is conceivable that o-acetylation precedes epimerisation which is followed by degradation. This is because o-acetylation blocks epimerisation and degradation while degradation is blocked by the other two modifications (Wong et al. 2000). Alternatively, alginate structure could result from the relative rates of three simultaneous reactions (Gacesa 1998).

1.5.3.1 O-acetylation

During its periplasmic journey, the nascent alginate is o-acetylated by AlgI, J and F (Figure 9); however, these proteins are not essential for alginate production. They add o-acetyl ester linkages on the O2/O3 position of M residues (Franklin & Ohman 2002, Franklin et al. 2004). The source of the acetyl group is currently unknown; however, acetyl-coenzyme A is the likely candidate (Gacesa 1998). The acetyl group is thought to be transported to the periplasm by AlgI, a cytoplasmic membrane protein with homologies (21% similarity) to a protein in *Bacillus subtilis*, Ipa-4r (DltB), which transports an activated precursor during lipoteichoic acid biosynthesis (Franklin & Ohman 1996). The second enzyme required for o-acetylation, AlgJ, is a periplasmic protein associated with the cytoplasmic membrane which is similar (30% identity and

69% similarity) to AlgX; both proteins have sugar-binding-hydrolysing domains likely to be involved in substrate binding (Robles-Price et al. 2004). The genes encoding AlgI and AlgJ are believed to be acquired by lateral gene transfer (Franklin et al. 2004). The third enzyme involved in o-acetylation, the periplasmic protein AlgF, does not have sequence homology to other proteins involved in o-acetylation (Franklin & Ohman 1996). Conserved residues in AlgI, J and F have been identified; however, the roles they play in o-acetylation are not clear (Franklin et al. 2004).

O-acetylation can restrict epimerisation and degradation, and o-acetylated alginate plays a pivotal role in the success of *P. aeruginosa* infections; approximately half of the residues of the alginate produced by the CF isolate *P. aeruginosa* FRD1 are o-acetylated (Franklin & Ohman 2002). In addition, o-acetylated alginate favours the development of microcolonies during biofilm growth which protects the pathogen from opsonic phagocytosis (Pier et al. 1994, Nivens et al. 2001).

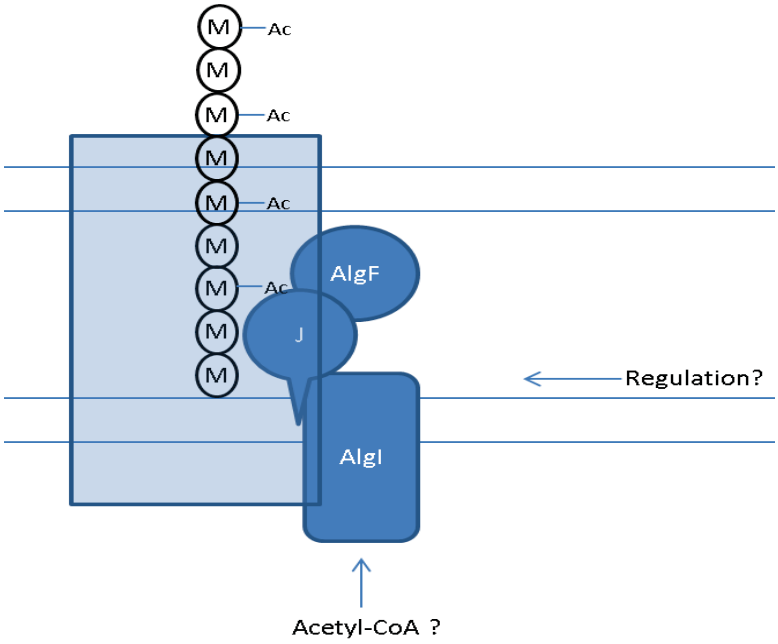

Figure 9. O-acetylation of alginate. During periplasmic translocation, the O2/O3 positions of mannuronate residues of the polymannuronate chain are o-acetylated (addition of –Ac) by AlgI, AlgJ and AlgF. AlgI is proposed to transfer the acetyl-donor molecule, thought to be acetyl-CoA, across the cytoplasmic membrane. The sugar-binding/hydrolysing domain of AlgJ could bind the alginate chain during o-acetylation. Although the proteins facilitating o-acetylation are dispensable for alginate production, o-acetylated alginate still plays a vital role in biofilm architecture and resistance against immune responses. Given this importance, o-acetylation could be tightly regulated.. Semi-transparent rectangle = alginate biosynthesis machinery.

1.5.3.2 Epimerisation

AlgG is a bi-function protein which specifically catalyses the epimerisation of M to G via protonation-deprotonation of C5 on a target residue; it also forms part of the periplasmic scaffold which protects the nascent alginate chain from AlgL degradation (Figure 8 & 10, Gimmestad et al. 2003, Jain et al. 2003, Douthit et al. 2005, Jerga et al. 2006b). Mutation of the catalytic residues of AlgG does not affect alginate yield, signifying that the epimerase function of AlgG is not essential for the synthesis of high molecular weight alginate (Gimmestad et al. 2003, Jain et al. 2003, Douthit et al. 2005). However, deletion of the *algG* gene results in the release of free uronic acid oligomers, indicating that AlgG protects the polymer from degradation (Gimmestad et al. 2003, Jain et al. 2003, Douthit et al. 2005).

The catalytic residues of AlgG reside in a shallow groove situated in a right-handed beta-helix fold (RHβH), a common motif of carbohydrate binding and sugar hydrolysing proteins (Jain et al. 2003, Douthit et al. 2005). *In vitro* studies demonstrate that AlgG has higher affinity to larger substrates up to 20 residues (100 Å long) – clearly too long for a single enzyme – signifying that several AlgG proteins may bind and epimerise alginate simultaneously (Jerga et al. 2006a). While an apparent equilibrium of 75% G content is reached when AlgG is incubated with poly-M substrate *in vitro*, the alginate synthesised by *P. aeruginosa* is only 50% G content, which could indicate that strict regulation and/or competition between modification pathways is occurring *in vivo* (Jerga et al. 2006a).

1.5.3.3 Degradation

Initially, it was somewhat intriguing to find an alginate lyase encoding gene, *algL*, residing within the alginate biosynthesis operon. AlgL specifically cleaves the alginate chain at poly-M residues via beta elimination, producing mannuronic acids with unsaturated non-reducing ends; the initial steps of this reaction are strikingly similar to epimerisation (Jerga et al. 2006b). Generation of *algL* mutants in mucoid strains is difficult, often resulting in non-viability or loss of mucoidity due to secondary mutations turning off alginate production (Bakkevig et al. 2005, Jain & Ohman 2005). A similar phenotype has been observed for strains producing AlgL with mutated

catalytic residues (Albrecht & Schiller 2005, Bakkevig et al. 2005). Several research groups have demonstrated that both AlgL and its lyase activity are required for viability in alginate over-producing strains, strongly suggesting that AlgL serves a maintenance role by degrading misguided alginate trapped in the periplasm (Albrecht & Schiller 2005, Bakkevig et al. 2005, Jain & Ohman 2005). However, at this stage, it is not clear whether AlgL is essential for the integrity of the periplasmic multi-protein scaffold or it remains a free periplasmic protein that degrades misguided alginate (Figure 10). This protein could also control polymer length (Bakkevig et al. 2005).

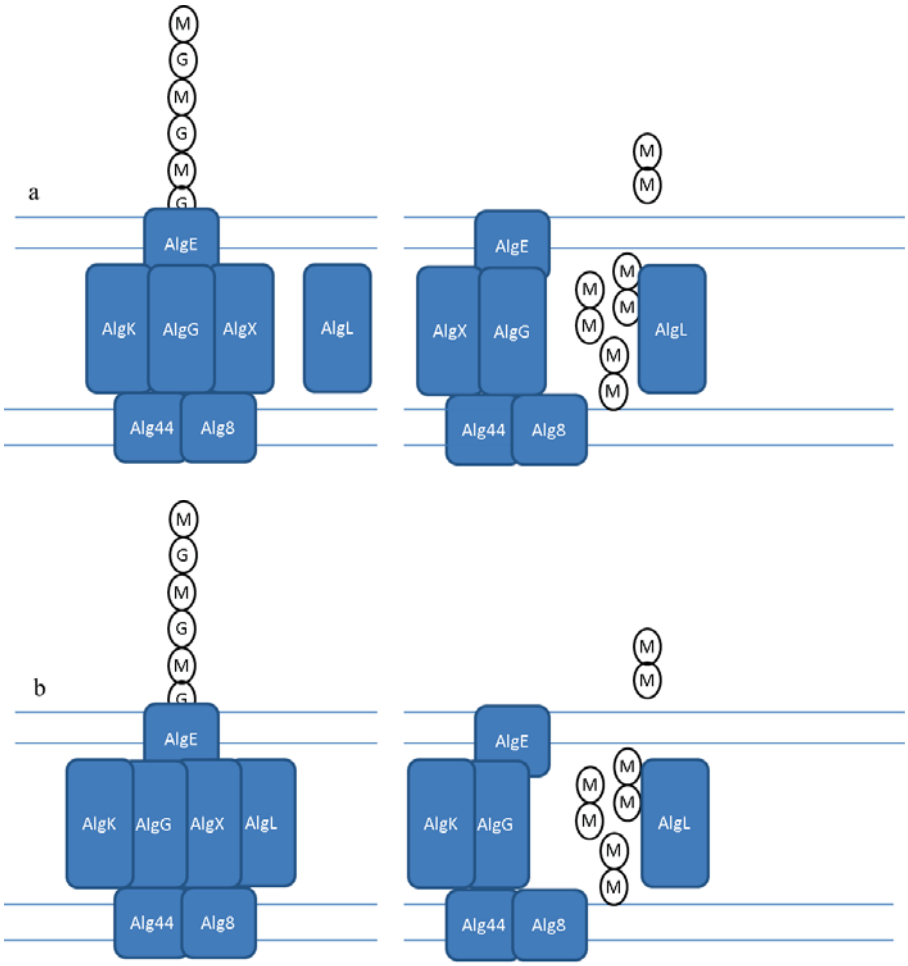

Figure 10. Periplasmic translocation, secretion and degradation of alginate. Alginate is translocated through the periplasm and secreted across the outer membrane by a multi-protein scaffold consisting of AlgK, AlgG, AlgX and AlgE. When any of these components is missing, alginate leaks into the periplasm where it is degraded by AlgL, an alginate lyase, releasing free uronic acid oligomers. According to Bakkevig et al. (2005), AlgL does not contribute to the scaffold integrity; instead, AlgL only has a maintenance role, degrading alginate trapped in the periplasm (a). In contrast, Jain & Ohman (2005) proposed that AlgL is bi-functional, forming part of the periplasmic multi-protein scaffold and degrading alginate trapped in the periplasm (b).

1.5.4 Secretion across the outer membrane

The outer membrane beta barrel porin, AlgE, is responsible for the secretion of mature alginate (Hay et al. 2010b). This protein displays anion selectivity upon spontaneous incorporation into planar lipid bilayers (Rehm et al. 1994). Recently, the crystal structure of AlgE has been determined (Whitney et al. 2011). Despite the lack of sequence similarity, AlgE is found to be structurally similar to OprD, a substrate specific nutrient uptake channel (Whitney et al. 2011). The constriction of the AlgE pore, formed by the extracellular loops L3 and L7 folding into the barrel cavity, is lined with charged and polar residues (Hay et al. 2010b, Whitney et al. 2011). The pore lining of AlgE is rich in arginine residues, similar to the substrate binding surface of an alginate lyase (A1-III from *Sphingomonas* sp) whose structure has been solved in complex with its substrate (Whitney et al. 2011). AlgE has an unusually long periplasmic loop (L8) which may interact with periplasmic domains of other subunits of the alginate biosynthesis machinery such as the TPR and/or MFP domains of AlgK and Alg44, respectively. The AlgK/E pair shares homology to enzymes involved in cellulose, Pel exopolysaccharide, and poly-β-1,6-N-acetyl-D-glucosamine (PGA) biosynthesis (Keiski et al. 2010). In addition, the functional homologues of AlgE and AlgK (HmsH and HmsF) facilitating PGA secretion in *Yersinia pestis* have been found to interact (Abu Khweek et al. 2010). Furthermore, the PGA secretion protein of *E. coli,* PgaA, represents a fusion of AlgE and AlgK (Itoh et al. 2008). The arrangements of subunits in the alginate biosynthesis machinery do not nicely fit into existing classes of polysaccharide polymerisation and secretion systems, suggesting that the AlgK/E pair may fall into a new class, strikingly different to existing ones (Keiski et al. 2010).

1.6 The role of AlgK in alginate production

AlgK's open reading frame, codon usage, GC (~70%) content and protein hydrophobicity were first characterised by Aarons et al. (1997). AlgK is localised to the periplasm after cleavage of its N terminal signal peptide sequence and subsequent lipidation (Aarons et al. 1997, Jain & Ohman 1998). The mature protein is anchored to the inner leaflet of the outer membrane by a lipid moiety (Keiski et al. 2010). Currently, it is proposed that AlgK facilitates periplasmic translocation of alginate; in the absence

of AlgK alginate is not produced, instead, free uronic acid oligomers presumably resulting from AlgL degradation are released (Jain & Ohman 1998).

AlgK has no known homologues at the primary structure level (Keiski et al. 2010). However, at the tertiary structure level, homologues predominantly belong to the TPR superfamily, including O-linked N-acetylglucosamine transferase from *Homo sapiens* and HcpC from *Helicobacter pylori* (Jinek et al. 2004, Luthy et al. 2004, Keiski et al. 2010). Recently, the crystal structure of AlgK has been solved (Keiski et al 2010). It was found to have multiple (9.5) tetratricopeptide-like (TPR-like) repeat motifs, a feature characteristic of proteins involved in the assembly of multi-protein complexes, suggesting that AlgK could be critical for assembling functional alginate biosynthesis machinery.

It has been shown that AlgK and Alg44 are required for the proper localisation of AlgE to the outer membrane, suggesting an interaction between these subunits (Oglesby et al. 2008, Keiski et al. 2010). AlgK has also been proposed to interact with the MFP domain of Alg44 as well as the outer membrane protein AlgE (Keiski et al. 2010, Whitney et al. 2011). However, direct experimental evidence is lacking. So far, only one direct protein-protein interaction between AlgK-AlgX-MucD has been demonstrated experimentally (Hay et al. 2012).

1.7 Aim and objectives of this study

On the basis of the current knowledge outlined in previous sections, the overall aim of this study was to investigate the role of AlgK in alginate biosynthesis with five objectives:

(1) To generate an isogenic marker free *algK* deletion mutant from a stable alginate over-producer, *P. aeruginosa* PDO300, for the elucidation of AlgK's role in alginate production.

(2) To complement this *algK* mutant by independently introducing *algK* ORF and *algK-his in trans* and *in cis*, generating four complemented strains

(3) To quantify high molecular weight (HMW) alginate and free uronic acid (FUA) oligomer yield of the strains generated to investigate the effect of AlgK absence, *algK* gene copy number and hexahistidine tag on alginate yield and ratio of HMW alginate to FUA oligomers.

(4) To investigate the role of AlgK in the stability of other subunits of the alginate biosynthesis machinery (e.g. AlgE, Alg44 and AlgX), and to assess whether the absence of various subunits would destabilise AlgK and each other through immunoblot analyses.

(5) To identify potential interaction partners of AlgK through pull down and immunoblot analyses.

1.8 Hypothesis statement

This study tested four hypotheses:

(1) An isogenic marker free *algK* deletion mutant can be generated which does not produce high molecular weight alginate.

(2) Introducing the *algK* ORF or *algK-his* can restore alginate biosynthesis.

(3) AlgK is required for the stability of AlgE, Alg44 and AlgX.

(4) AlgK interacts directly with components of the alginate biosynthesis machinery (e.g. AlgE, Alg44, AlgX).

CHAPTER TWO

MATERIALS AND METHODS

Unless stated otherwise, reagents were purchased from Sigma, Ajax Finechem or Merck; all centrifugation events were carried out at room temperature (22 ± 4°C), and 'water' or 'H_2O' referred to autoclaved Milli-Q water. When required the absorbance of samples was measured using a Biochrom Libra S5 spectrophotometer (Biochrom Ltd, England).

2.1 Strains, plasmids and oligonucleotides

Tables 2, 3 and 4 outline the bacterial strains, plasmids and oligonucleotides used in the present study. All oligonucleotide primers were synthesised by Sigma-Aldrich, USA.

TABLE 2. Bacterial strains used in this study

Strains	Description	Sources or references
P. aeruginosa PDO300	Δ*mucA22* variant of PAO1; Alg+	(Mathee et al. 1999)
P. aeruginosa PDO300Δ*algK*ΩGm	Δ*algK::aacC1*; Alg-	This study
P. aeruginosa PDO300Δ*algK*	Δ*algK*; Alg-	This study
P. aeruginosa PDO300Δ*algE*	Δ*algE*; Alg-	(Hay et al. 2010b)
P. aeruginosa PDO300Δ*alg44*	Δ*alg44*; Alg-	(Remminghorst & Rehm 2006a)
P. aeruginosa PDO300Δ*algX*	Δ*algX*; Alg-	(Gutsche et al. 2006)
E. coli TOP10	Cloning strain	Invitrogen
E. coli S17-1	thi-1 proA hsdR17 (Rk-Mk+) recA1; tra gene of plasmid RP4 integrated in chromosome	(Simon et al. 1983)

TABLE 3. Plasmids used in this study

Plasmids	Description	Sources or references
pGEM®-T Easy	Ap^R cloning plasmid (referred to as pGEMt-Easy in text)	Promega
pGEMt-*algKNC*ΩGm	*algKN*-FRT-*aacC1*-FRT-*algKC* inserted in pGEMt-easy	This study
pBBR1MCS-5	Gm^R; broad-host-range vector; P*lac*	(Kovach et al. 1995)
pBBR1MCS-5:*algK*	*Hind*III-*Bam*HI fragment comprising *algK* gene inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*algK6-his*	*Hind*III-*Bam*HI fragment comprising *algK-his* inserted into vector pBBR1MCS-5	This study
pEX100T	Ap^R, Cb^R, gene replacement vector containing *sacB* gene for counter-selection	(Hoang et al. 1998)
pEX100TΔ*algK*ΩGm^R	Ap^R, Cb^R, Gm^R, vector with *Sma*I-inserted *algK* deletion construct	This study
pPS856	Ap^R, Gm^R, source of 1,100bp *Bam*HI fragment comprising *aacC1* flanked by FRT recognition sequences	(Hoang et al, 1998)
pFLP2	Ap^R, Cb^R; broad-host-range vector encoding Flp recombinase	(Hoang et al. 1998)
miniCTX-2	Self-proficient integration vector Tet^R	(Hoang et al. 2000)
miniCTXP*alg*:*algK*	Self-proficient integration vector, *algK* gene under control of *algD* promoter	This study
miniCTXP*alg*:*algK-his*	Self-proficient integration vector, *algK-his* under control of *algD* promoter	This study
miniCTXP*alg*:*algE*	Self-proficient integration vector, *algE* under control of *algD* promoter	(Rehman et al. 2013)
miniCTXP*alg*:*alg44*	Self-proficient integration vector, *alg44* under control of *algD* promoter	(Rehman et al. 2013)
miniCTXP*alg*:*algX*	Self-proficient integration vector, *algX* under control of *algD* promoter	(Rehman et al. 2013)

TABLE 4. Oligonucleotide primers used in this study

Name	Oligonucleotide sequence (5' to 3')	Sources or references
*algK*NF(EcoRV)	GATATCATGAAGATGCCCATCCTCCCTCC	This study
*algK*NR(BamHI)	GGATCCCGATTTCCGGCCAGGACTGC	This study
*algK*CF(BamHI)	GGATCCTACTACCTGGGGCAGATCTA	This study
*algK*CR(EcoRV)	GATATCCTCATAGGCTTTCTGGCTCTTC	This study
algK(upXout)	ACCCTGCTGAACAAGGCCGTGAC	This study
algK(downXout)	GCGGGTTGACGGAACGGGAGCTG	This study
*algK*N(HiSDNd)	ACCAAAGCTTAGGAGAGAAAGCATATGAAGATGCC CATCCTCCCTCCCCTGC	This study
*algK*C(BamHI)	GACGGATCCTCATAGGCTTTCTGGCTCTTCTTCG	This study
*algK*C(x6-hisBamHI)	GTCTGGGATCCGACTTAATGATGGTGATGGTGGTG TAGGCTTTCTGGCTCTTCTTCGTTGATCGGCGAG	This study
Pser Up	CGAGTGGTTTAAGGCAACGGTCTTGA	(Hoang et al. 2000)
Pser Down	AGTTCGGCCTGGTGGAACAACTCG	(Hoang et al. 2000)
PstAlgPF	GCACTGCAGGCGGCCGCCTCCTCTTTCGG	This study
HiIIIAlgPR	ACAAAGCTTGCATTCACCTCGATTGTTTG	This study

2.2 Media

The following liquid media were used in this study. Solid media were prepared by adding agar (Neogen, USA) to 1.5% (w/v) prior to autoclaving. All media were autoclaved at 121°C for 20 min. When required, antibiotics (Section 2.3) were added after autoclaving (Table 5).

2.2.1 Luria-Bertani (LB) medium

Luria-Bertani (LB) medium (Invitrogen Corporation, USA) was prepared according to the manufacturer's instructions. Twenty grams of dry media was dissolved in 1 litre of water.

2.2.2 X-Gal medium

This medium was prepared by adding the following reagents to the LB medium (Section 2.2.1) after autoclaving:

1M IPTG	1.0	ml
4% (w/v) X-Gal (in N,N'-Dimethylformlamide)	1.0	ml

2.2.3 *Pseudomonas* isolation (PI) medium

Both liquid *Pseudomonas* isolation (PI) and solid PI agar (PIA) were used in the present study. The former was prepared as described by Remminghorst & Rehm (2006b):

Peptone	20.0	g
K_2SO_4	10.0	g
$MgCl_2 \times 6\ H_2O$	1.4	g
Triclosan	25.0	mg
Glycerol	20.0	ml
H_2O	1000	ml
pH	7.0	

The latter was prepared according to the manufacturer's instructions (Becton, Dickinson and Company, USA). Forty five grams of dry media was added to 1 litre of water and supplemented with glycerol to 2% (v/v) and agar (Neogen, USA) to 1.5% (w/v) before autoclaving.

2.2.4 Nutrient Broth

Nutrient Broth (Oxoid, England) was prepared according to the manufacturer's instructions by adding 13 g of dry media to 1 litre of water.

2.2.5 Mineral salt medium (MSM)

Mineral salt medium (MSM) was prepared as described previously (Schlegel et al. 1961):

$Na_2HPO_4 \times 12\ H_2O$	9.0	g
KH_2PO_4	1.5	g
NH_4Cl	1.0	g
$MgSO_4 \times 7\ H_2O$	0.2	g
$CaCl_2 \times 2\ H_2O$	0.02	g
Fe(III)NH_4-Citrate	1.2	mg
Supplement solution SL6	0.1	ml
H_2O to	1000	ml
pH	7.5	

The following outlines the composition of supplement solution SL6 as described by Schlegel et al. (1961):

$ZnSO_4 \times 7\ H_2O$	10.0	mg
$MnCl_2 \times 4\ H_2O$	3.0	mg
H_3BO_3	30.0	mg
$CoCl_2 \times 6\ H_2O$	20.0	mg
$CuCl_2 \times 2\ H_2O$	1.0	mg
$NiCl_2 \times 6\ H_2O$	2.0	mg

$Na_2MO_4 \times 2\ H_2O$	3.0	mg
H_2O to	1000	ml

When necessary, sucrose was added to a final concentration of 5% (w/v) from a sterile stock solution of 40% (w/v), prepared via filtration through a 0.22 µm filter. To prepare 1 litre of solid MSM agar, components for 1 litre of MSM were solubilised in 0.5 litre of water while an agar solution of 3% (w/v) was prepared in 0.5 litre of water. After autoclaving, the solutions were cooled to approximately 50°C and combined.

2.3 Antibiotic stock solutions and concentrations

Antibiotic stock solutions were prepared as listed in Table 5.

TABLE 5. Antibiotic stock solutions and concentrations

Antibiotics	**Stock solutions (mg/ml)**	**Final concentration (µg/ ml)**
	For *E. coli*	
Ampicillin (Na-salt)	75 in H_2O	75
Gentamycin (sulphate)	10 in H_2O	10
Tetracycline (hydrochloride)	12.5 in EtOH 70% (v/v)	12.5
	For *P. aeruginosa*	
Gentamycin (sulphate)	300 in H_2O	300
Tetracycline (hydrochloride)	200 in H_2O	200
Carbenicillin	300 in H_2O	300

2.4 Cultivation conditions

All bacterial strains were cultivated at 37ºC. Liquid cultures were grown in Erlenmeyer flasks or universal tubes with shaking at 200 rpm. A ratio of container to culture volume of ≥5:1 was maintained to ensure adequate aeration.

2.5 Long term storage of strains

E. coli and *P. aeruginosa* strains were grown overnight in LB (Section 2.2.1) and PI (Section 2.2.3) media, respectively, supplemented with appropriate antibiotics (Section 2.3). One millilitre of culture was transferred to a sterile 1.8 ml cryotube vial (Thermo Scientific, USA) and 70 µl of DMSO was added. Strains were stored at -80ºC and revived when required.

2.6 Competent cell preparation and plasmid DNA uptake

Preparation and transformation of competent *E. coli* were carried out as outlined by Hanahan (1983). The methods of Choi & Schweizer (2006) and Friedrich et al. (1981) were used for the electro-poration and transconjugation of *P. aeruginosa*, respectively.

2.6.1 Preparation and transformation of competent *E. coli*

The relevant *E. coli* strain was grown in 50 ml of LB (Section 2.2.1), with appropriate antibiotics (Section 2.3), to an optical density at 600 nm ($O.D_{600nm}$) of 0.3. The culture was incubated on ice for 10 min before cells were harvested via centrifugation (4,000 g at 4ºC for 20 min). The cell pellet was re-suspended in 18 ml of RF1 solution and incubated on ice for 1 h. The cells were spun down as above and re-suspended in 4 ml of RF2 solution. Two hundred microliter aliquots were transferred into clean sterile 1.7 ml microcentrifuge tubes, flash frozen in liquid nitrogen and stored at -80ºC. Below outlines the composition of RF1 and RF2 solutions which were sterilised by filtration through separate 0.22 µm filters:

RF1 solution:

RbCl	100.0 mM
$MnCl_2$	50.0 mM
Potassium acetate	30.0 mM
$CaCl_2 \times 6\ H_2O$	10.0 mM
	Adjusted to pH 5.8 with acetic acid

RF2 solution:

RbCl	10.0 mM
MOPS	10.0 mM
$CaCl_2 \times 6\ H_2O$	75.0 mM
Glycerol	15.0% (v/v)
	Adjusted to pH 5.8 with NaOH

For the transformation of *E. coli*, 50 to 500 ng of plasmid DNA was added to frozen competent cells (as prepared above) and incubated on ice for 1 h. Cells were subject to heat shock (42°C for 90 s) and returned to ice for 5 min. Eight hundred microliters of LB medium (Section 2.2.1) was added to the cells and incubated for 1 h at 37°C. Cells were harvested by centrifugation (15,000 g for 2 min) and the cell pellet was re-suspended in 200 µl of LB medium and plated on LB agar containing appropriate antibiotics (Section 2.3) to select for colonies containing the plasmid of interest.

2.6.2 Preparation and electroporation of electro-competent *P. aeruginosa*

Preparation and electroporation of electro-competent *P. aeruginosa* were performed as described by Choi & Schweizer (2006). The relevant *P. aeruginosa* strains were grown overnight in 20 ml of LB medium (Section 2.2.1) supplemented with antibiotics (Section 2.3). Six millilitres of the overnight culture was equally distributed into four microcentrifuge tubes and the cells were harvested by centrifugation (15,000 g for 2 min). The cells were washed twice in 300 mM sterile sucrose solution and harvested by centrifugation. The cells from the four tubes were combined into one microcentrifuge tube and suspended in 100 µl of 600 mM sterile sucrose solution. This suspension of electro-competent *P. aeruginosa* cells was mixed with 100 µl of water containing 250-500 ng of plasmid DNA, giving a final sucrose concentration of 300 mM. This suspension was transferred to a 2 mm gap Gene Pulser electroporation cuvette (Biorad, USA) and after applying pulse (2.5 kV) using a Biorad Micropulser (Biorad, USA), 800 µl of LB medium (Section 2.2.1) was added. The culture was then transferred to a sterile 15 ml universal tube and allowed to recover at 37°C for 1.5 h with shaking at 200 rpm. Due to the high efficiency of electroporation of medium copy number plasmids

(pBBR1MCS-5 derivatives), cell suspensions receiving such plasmids were diluted 4,000-fold before plating onto solid PIA (Section 2.2.3.1) containing appropriate antibiotics (Section 2.3) to select for successful transformants.

2.6.3 Transconjugation of *P. aeruginosa*

Transfer of conjugation-competent-plasmids into *P. aeruingosa* strains was performed as outlined previously (Friedrich et al. 1981). The donor strains *E. coli* S17-1 and SM10 were used to transfer plasmids harbouring the Mob (*oriT*) region into *P. aeruginosa* strains. The plasmid of concern was transformed into the *E. coli* donor strain (Section 2.6.1). This strain and the recipient *P. aeruginosa* strain were grown separately in 20 ml of LB medium (Section 2.2.1) to mid log ($O.D_{600nm}$ of 0.6-0.8) and mid stationary phases (24 h), respectively. Cultures were harvested via centrifugation (4,000 g at 4°C for 20 min) and the cell pellets were thoroughly suspended in 0.9% (w/v) sterile saline and centrifuged under the same conditions. Cells were re-suspended in 1 ml of sterile saline and 200 µl of the donor and recipient suspensions were combined in sterile 1.7 ml micro-centrifuge tubes. After mixing by inversion, the cells were harvested via centrifugation (15,000 g for 2 min), re-suspended in 200 µl of sterile saline and then carefully poured onto an NB agar plate (Section 2.2.4). After five minutes, the plates were gently transferred to 37°C and incubated for 24 h. After incubation, the cells were scrapped from plates using a sterile spatula, and re-suspended in 1 ml of sterile saline. After a 1,000-fold dilution (no dilution required for pEX100T derived plasmids), 100 µl of the cell suspension was plated on MSM agar (Section 2.2.5) containing appropriate antibiotics (Section 2.3) and carbon source to select for successful tranconjugants.

2.7 DNA isolation, analysis and manipulation

General protocols for DNA manipulation were performed (Sambrook et al. 1989).

2.7.1 Isolation of chromosomal DNA

Chromosomal DNA of *P. aeruginosa* was isolated as described by Mak & Ho (1992). *P. aeruginosa* strains were grown overnight in 50 ml of LB medium (Section 2.2.1) and cells were harvested via centrifugation (4,000 g at 4°C for 20 min). Cells were re-suspended in 2 ml of LB medium containing 0.1% (w/v) SDS. Two hundred and fifty

microliters of this suspension was then mixed with 250 µl of LB-SDS medium in a 1.7 ml micro-centrifuge tube. Next, 500 µl of phenol was added and the solution was left on a horizontal shaker for 4 h. After shaking, cell debris was removed via centrifugation (15,000 g for 10 min). The DNA containing aqueous (top) phase was combined with 0.5 volumes of phenol and 0.5 volumes of chloroform/isoamylalcohol solution (19:1 v/v) in a micro-centrifuge tube and thoroughly vortexed and centrifuged as above. The aqueous layer was transferred to a micro-centrifuge tube and the DNA was precipitated with one volume isopropanol. After centrifugation (15,000 g for 20 min), the DNA pellet was washed twice with 70% (v/v) ethanol and air dried, dissolved in TE-RNase-A (20 mg/ml RNase-A, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and stored at -20°C.

2.7.2 Isolation of plasmid DNA

The High Pure Plasmid Isolation Kit (Roche, USA) was used for isolation of plasmid DNA from cell cultures. Briefly, cells containing the plasmid of interest were grown at 37°C for 16 h in LB medium (Section 2.2.1) supplemented with antibiotics (Section 2.3). Cells were harvested and suspended in the Suspension Buffer containing RNase and disrupted by alkaline lysis using the Lysis Buffer. Upon lysis, the RNase removed RNA contamination. To the lysed cell suspension, the Binding Buffer was added. The chaotrophic salt, guanidinium chloride, present in the Binding Buffer denatured the proteins, which precipitated while entrapping the chromosomal DNA. After centrifugation (15,000 g for 10 min) to remove the precipitated proteins and chromosomal DNA, the plasmid DNA of the soluble fraction was bound to the glass fleece of the High Pure Filter Tube. After washing the High Pure Filter Tube with Wash Buffers I and II to remove contaminants, 100 µL of the Elution Buffer (10 mM Tris-HCl buffer pH 8.5) was used to elute the plasmid DNA. For isolation of plasmid DNA from *P. aeruginosa*, cells were washed twice with sterile saline to remove excess extracellular material prior to suspension in the Suspension Buffer.

2.7.3 Isolation of linear dsDNA

To isolate linear dsDNA from polymerase chain reaction (Section 2.7.6) or restriction endonuclease hydrolysis (Section 2.7.7), DNA was first subject to agarose gel electrophoresis (Section 2.7.5) supplemented with the dsDNA stain SYBR green at ×1

concentration (Invitrogen Corporation, USA). Under long wavelength UV light, the band corresponding to the target dsDNA fragment was excised. Linear dsDNA was purified from the gel fragment using the PureLink® Gel Extraction Kit according to the manufacturer's instructions (Invitrogen Corporation, USA). Briefly, the excised gel fragment containing the linear dsDNA of interest was solubilised in Solubilisation Buffer (SB) at 50°C. After solubilising the gel, one gel volume of isopropanol was added. DNA from the sample was then bound to the Spin Column and washed once using the supplied wash buffer to remove contaminants. Finally, the dsDNA was eluted using the provided elution buffer.

2.7.4 Determination of DNA concentration and purity

DNA concentration and purity were assessed by spectrophotometry, fluorescence and quantitative gel electrophoresis. For spectrophotometric analysis, an absorbance ratio at 260/280 nm of 1.8 to 2.0 indicates high purity DNA (Nano-drop ND-1000, USA). The Qubit™ fluorometer (Invitrogen Corporation, USA) was used in conjunction with Quant-iT DNA BR Assay Kit (Invitrogen Corporation, USA) for accurate determination of DNA concentration (emission/excitation wavelength of ~480/530 nm). This method appeared to be more reliable than spectrophotometric analysis using Nano-drop ND-1000; however, it was unable to assess contaminant concentration. The concentration and purity of linear dsDNA fragments were also estimated via quantitative gel electrophoresis. In this method, the clarity and intensity of DNA bands of serially diluted DNA samples and MassRuler High Range DNA ladder (Fermentas, USA) were compared.

2.7.5 Agarose gel electrophoresis (AGE)

AGE was used to determine DNA concentration and purity (Section 2.7.4), and separate DNA fragments after polymerase chain reaction (Section 2.7.6) and restriction endonuclease hydrolysis (Section 2.7.7). When required, DNA could be recovered from gels (Section 2.7.3). In general, agarose gels of 1% and 2% (w/v) were used to resolve dsDNA fragments above and below 500 bp, respectively. Agarose (Bioline, USA) was combined with TBE electrophoresis buffer (50 mM Tris-HCl, 50 mM Boric acid, 2.5 mM EDTA, pH 8.0) and melted in microwave oven, poured into a gel chamber and a

well comb inserted. After solidification (1 h), the comb was removed and the gel chamber, together with gel, was placed into an electrophoresis apparatus and submerged in TBE buffer. DNA samples supplemented with 0.2 vol of 6× stop-mix (loading dye) were loaded into wells. The composition of 6× stop-mix is outlined below:

Tris-HCl	60.0	mM
EDTA	60.0	mM
Glycerol	60.0	% (v/v)
Orange G	0.2	% (w/v)
Xylene Cyanol FF	0.05	% (w/v)

A molecular weight standard was loaded into a separate well. DNA molecular weight standards used in this study were Lambda phage DNA hydrolysed with the restriction endonuclease *Pst*I (Sambrook et al. 1989) and MassRuler High Range DNA ladder (Fermentas, USA). In general, gels were run in TBE electrophoresis buffer at 6-7 V/cm - distance between anode and cathode - for 30-60 min. Gels were stained for 15 min in ethidium bromide solution (2 µg/ml) and de-stained for 1 min in water. DNA bands were visualised using an UV transilluminator (Bio-Rad, Gel Doc, USA) and images were generated.

2.7.6 Polymerase chain reaction (PCR)

Where PCR was performed to obtain fragments for cloning, the high fidelity proofreading Platinum® *Pfx* DNA polymerase (Invitrogen Corporation, USA) was used. Where the amplification was for diagnostic/verification purposes, Platinum® *Taq* DNA polymerase (Invitrogen Corporation, USA) was employed. Reaction mixtures were prepared as outlined below in 0.2 ml clean sterile thin walled PCR tubes (Axygen, USA):

Pfx-DNA-polymerase reaction mixture (A)

10X *Pfx* Amplification Buffer	20.0	µl
PCR_x Enhancer Solution	22.5	µl
$MgSO_4$ (50 mM)	2.0	µl
DMSO	2.5	µl
Primer 1 (10 pmoles/µl)	3.0	µl
Primer 2 (10 pmoles/µl)	3.0	µl
dNTPs (10 mM each)	3.0	µl
Template DNA	~2.0	ng
Platinum® *Pfx* DNA Polymerase (2.5 U/ µl)	1.0	µl
H_2O	to 100	µl

Pfx-DNA-polymerase reaction mixture (B)

10X *Pfx* Amplification Buffer	20.0	µl
$MgSO_4$ (50 mM)	5.0	µl
DMSO	5.0	µl
Primer 1 (10 pmoles/µl)	10.0	µl
Primer 2 (10 pmoles/µl)	10.0	µl
dNTPs (10 mM each)	10.0	µl
Template DNA	~2.0	ng
Platinum® *Pfx* DNA Polymerase (2.5 U/ µl) 0.5	µl	
H_2O	to 100	µl

***Taq*-DNA-polymerase reaction mixture**

Glycerol 50% (v/v)	20.0	µl
DMSO	5.0	µl
10X PCR Buffer (minus $MgCl_2$)	10.0	µl
$MgCl_2$ (50 mM)	10.0	µl
Primer 1 (10 pmoles/µl)	5.0	µl
Primer 2 (10 pmoles/µl)	5.0	µl
dNTPs (10 mM each)	10.0	µl
Template DNA	~2.0	ng
Platinum® *Taq* DNA Polymerase (5 U/µl)	1.0	µl
H_2O	to 100	µl

The PCR reactions were performed in a Biometra Tpersonal thermocycler (Whatman Biometra, Germany) as described below:

1) Primary denature: 94°C (*Pfx*) or 95°C (*Taq*) for 300 s
2) Denature: 94°C (*Pfx*) or 95°C (*Taq*) for 45 s
3) Anneal: ~5°C below the lowest Tm of the primer pair for 30 s
4) Extend: 68°C (*Pfx*) or 72°C (*Taq*) for 60 s per 1 kbp
5) Cycle: steps 2-4 for 30 cycles
6) Hold: 10°C

2.7.7 DNA hydrolysis with restriction endonucleases

Plasmid DNA (Section 2.7.2) and PCR products (Section 2.7.6) were hydrolysed by restriction endonucleases for cloning and analysis/verification purposes. Various restriction endonuclease enzymes (RE) were used according to the manufacturer's instructions (Invitrogen Corporation, Roche or New England Biolabs). For preparative hydrolysis, 100 µg of DNA was digested with 100 U of enzyme in water containing 0.1 vol of 10X recommended buffer. For restriction analysis, 10 U of enzyme was used to hydrolyse 1 µg of DNA in water containing 0.1 vol of 10X recommended buffer. When recommended by the manufacturer, bovine serum albumin was added to reactions to a final concentration of 10 mg/ml. All digestions were performed at 37°C for 2-4 h and stopped by adding 0.2 vol of 6× stop-mix. DNA fragments were separated via agarose gel electrophoresis (Section 2.7.5) and fragments of interest could be recovered from gels for subsequent cloning (Section 2.7.3).

2.7.8 pGEM®-T Easy Vector System: A-tailing, ligation and confirmation

The pGEM®-T Easy Vector System was used for amplifying DNA fragments (Promega, USA). The supplied cloning vector, pGEM®T Easy, herein referred to as the pGEMt-Easy vector, is a high copy number plasmid commonly used for cloning and sequencing. The pGEMt-Easy plasmid was supplied in a linear form, which has 5' thymidine overhangs (T-tails); permitting effective sticky-ended-ligation with double

stranded DNA fragments carrying 3' adenosine overhangs (A-tails). Figure 11 outlines the general strategy for cloning DNA fragments using this system.

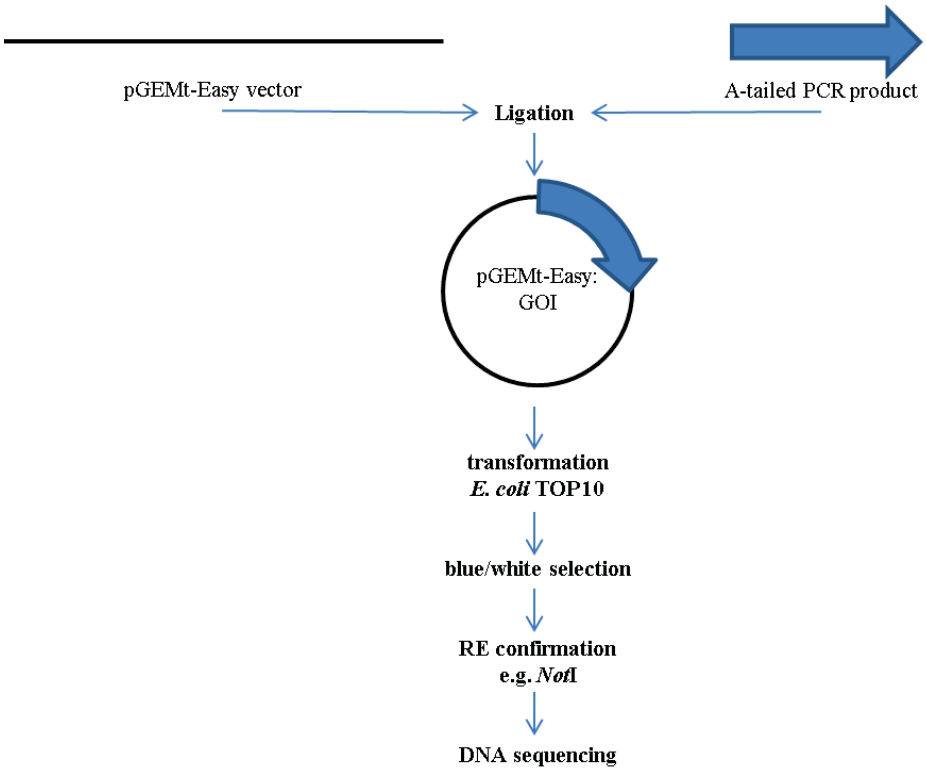

Figure 11. General cloning strategy using pGEM®-T Easy Vector System. Linear pGEM®-T Easy vector (referred to as pGEMt-Easy vector) was ligated with an A-tailed PCR product forming the plasmid pGEMt-Easy:GOI (gene of interest) which was propagated in *E. coli* TOP10 on X-Gal media containing ampicillin. White colonies were used to inoculate LB amp and grown overnight. Plasmid DNA isolated from cultures was screened by restriction endonuclease digestion and confirmed by DNA sequencing. Sequence-confirmed DNA fragments were excised from the plasmid using relevant restriction endonucleases, and gel purified and quantified ready for downstream cloning applications.

2.7.8.1 A-tailing

The PCR product (Section 2.7.6) was recovered from AGE (Section 2.7.3) and A-tailed using Platinum® *Taq* polymerase (Invitrogen Corporation, USA). This enzyme was used because it lacks a 3' to 5' exonuclease proofreading activity, making it prone to leaving 3' adenonsine overhangs. A-tailing reactions were prepared in 0.2 ml clean sterile thin walled PCR tubes (Axygen, USA) containing 6 µl of the recovered DNA

fragment (Section 2.7.3), 1 µl of 10X PCR buffer (minus $MgCl_2$), 1 µl of 50 mM $MgCl_2$, 1 µl of Platinum® *Taq* polymerase (5 U/µl) and 1 µl of 20 mM dATP. Reactions were subject to the temperature cycle of 95ºC for 5 min and then 70ºC for 30 min utilising the identical PCR machine (Section 2.7.6). For large scale A-tailing, the volumes of reagents and substrates were increased proportionally to give a final reaction volume of 100 µl.

2.7.8.2 Ligation

Ligation reactions of A-tailed PCR products (Section 2.7.8.1) with pGEMt-Easy vector were prepared in 0.2 ml clean sterile thin walled PCR tubes (Axygen, USA) containing 1 µl of T4 DNA ligase, 5 µl of 2X ligase buffer, 0.5 µl of pGEMt-Easy vector (50 ng/µl), 1 to 3.5 µl of A-tailed DNA fragment (Section 2.7.8.1) as required to give a vector to insert molar ratio of 1:6, and water to a final volume of 10 µl. Reactions were incubated for 16 h at 4ºC. All enzymes and buffers were supplied by Promega, USA. In all ligations, DNA concentration was kept between 5 and 10 ng/µl.

2.7.8.3 Confirmation

In general, pGEMt-Easy derived plasmids were propagated in *E. coli* TOP10 (Invitrogen, USA) (Section 2.6.1) on X-Gal media (Section 2.2.2) supplemented with appropriate antibiotics (Section 2.3). Successful ligation of the A-tailed PCR product into pGEMt-Easy was confirmed by blue/white selection, restriction digest with *Not*I (Section 2.7.7) (or other relevant enzymes) and DNA sequencing (Section 2.7.10).

2.7.9 DNA ligation with non-pGEM®-T Easy vectors

Insert DNA obtained from PCR (Section 2.7.6) or RE digestions (Section 2.7.7) was recovered from AGE (Section 2.7.3). Ligation reactions of insert with linearised vector were prepared in 0.2 ml clean sterile thin walled PCR tubes (Axygen, USA) containing 1 µl of T4 DNA ligase and 4 µl of 5× ligation buffer, and insert and vector DNA as required to give a molar ratio of 6:1. DNA concentration was kept between 5 to 10 ng/µl. Water was added to give a final vol of 20 µl. For efficient ligation, Reactions were incubated at 4°C for 16 h.

2.7.10 DNA sequencing

All DNA sequencing of recombinant plasmids was provided by the Massey Genome Service utilizing a capillary ABI3730 Genetic Analyser (Applied Biosystems Inc., USA). Results were provided in ABI format and analysed using Vector NTI version 11 (Invitrogen Corporation, USA). Four to six hundred nanograms of plasmid DNA purified from cell cultures (Section 2.7.2) was suspended in 14 µl of water and sent for sequencing in 0.2 ml clean sterile thin walled PCR tubes (Axygen, USA). Commercial primers (SP6 and T7) were used for sequencing pGEMt-Easy derived plasmids.

2.8 Assessment of general physiology

The methods for assessing growth rate in liquid media (Section 2.8.1) and colony morphology on solid media (Section 2.8.2) are presented here.

2.8.1 Growth rate

P. aeruginosa strains were grown in 50 ml of PI (Section 2.2.3) pre-cultures under standard cultivation conditions (Section 2.4) with antibiotics (Section 2.3). This was used to inoculate fresh PI media containing antibiotics to a final vol of 50 ml with an O.D $_{600nm}$ of 0.05. This culture was incubated at 37°C for 27 h with shaking at 200 rpm. O.D $_{600nm}$ was measured hourly for the first 16 h and then every 3-5 hours until 27 h was reached.

2.8.2 Colony morphology

P. aeruginosa strains were inoculated from frozen stocks and grown overnight in 5 ml of liquid PI media (Section 2.2.3), then streaked onto solid PIA media (Section 2.2.3), supplemented with antibiotics (Section 2.3), and incubated for 36 h. Colonies were photographed using a digital five megapixel camera (Canon Power Shot G5, Japan).

2.9 Characterisation of alginate production

High molecular weight alginate yield was measured for strains grown on solid PIA media (Section 2.2.3). Uronic acid size distribution (high molecular weight alginate vs. alginate degradation products) was measured for strains grown in liquid PI media (Section 2.2.3). Samples were prepared from solid (Section 2.9.1) and liquid (Section 2.9.2) media and uronic acid content was determined (Section 2.9.3).

2.9.1 Preparation of alginate sample from solid media

Alginate samples from strains cultivated on solid media were prepared as described by Remminghorst & Rehm (2006a). Frozen stock (Section 2.5) was used to inoculate solid PIA medium (Section 2.2.3) containing antibiotics (Section 2.3). After 24 h incubation, a single colony from this plate was used to inoculate 20 ml of liquid PI medium containing antibiotics. After 14 h incubation, 3 ml of this culture was equally distributed into three clean sterile microcentrifuge tubes. After centrifugation (15,000 g for 2 min), each cell pellet was washed twice with 1 ml of sterile saline, centrifuged as above and then re-suspended in 150 µl of sterile saline. Each cell suspension was evenly spread onto an individual PIA agar plate containing antibiotics. After 72 h incubation, biomass from plates was scrapped off, separately suspended in 150 ml of sterile saline and left on a horizontal shaker for 30 to 60 min as required for the biomass to enter suspension. After solubilising the biomass, cells were harvested by centrifugation (9,000 g at 4°C for 45 min) and the alginate in the supernatant was precipitated with 1 vol of ice-cold isopropanol. Next, the cell pellets and crude alginate precipitates were freeze-dried. For freeze drying, samples were stored at -80°C for 24 h and then dried at room temperature for 24 h using a floor-model freeze-dryer (Dura-Dry MP Model # FD2085C0000, FTS Systems, USA). Freeze-drying dehydrates the sample by sublimating the frozen water under reduced atmospheric pressure. The freeze-dried crude alginate samples were individually solubilised in buffer (0.05 M Tris-HCl, 10 mM $MgCl_2$) to a final concentration of 0.5% (w/v) and incubated with DNase I (15 µg/ml) and RNase A (15 µg/ml) for 6 h at 37°C with shaking at 200 rpm to remove nucleic acids. After a further 18 h incubation with Pronase E (20 µg/ml) under identical conditions to remove protein contamination, the samples were dialysed (12,000 kDa) against 5 litres of water for 24 h at 4°C. The enriched alginate was precipitated with ice-cold isopropanol, freeze-dried

and weighed. These samples were dissolved in 200 µl of water at 250 and 500 µg/ml and uronic acid content was determined (Section 2.9.3).

2.9.2 Preparation of alginate sample from liquid media

Alginate samples from strains grown in liquid media were prepared as described previously (Remminghorst & Rehm 2006a). Frozen stock (Section 2.5) was used to inoculate PIA medium (Section 2.2.3.1) containing antibiotics (Section 2.3). After 24 h incubation, a single colony from this plate was used to inoculate 5 ml of PI medium (Section 2.2.3) containing antibiotics. After 14 h incubation, this pre-culture was used to inoculate 50 ml of PI media, supplemented with appropriate antibiotics, to a final $O.D_{600nm}$ of 0.05. After 24 h incubation, 2 ml of culture was centrifuged (15,000 g for 10 min) and the cell pellet was freeze dried as described in Section 2.8.1 and weighed. Meanwhile, half of the supernatant was filtered using a Vivaspin 500, 10 kDa MWCO spin column (GE Healthcare, UK) by centrifugation (15,000 g at 4 °C for 1 h). Uronic acid content of the unfiltered supernatant and the filtrate was determined (Section 2.9.3). As a control for the background uronic acid content present in the media, uronic acid content of PI media before and after filtration was also determined (Section 2.9.3).

2.9.3 Uronic acid assay

Uronic acid content of samples was determined using a modified colorimetric assay described by Blumenkrantz & Asboe-Hansen (1973). Alginic acid sodium salt from brown seaweed was used as a standard (Sigma-Aldrich, USA). Two hundred microliter samples, prepared according to Sections 2.9.1 and 2.9.2, were added to 1.2 ml tetraborate solution (0.0125M tetraborate in concentrated sulphuric acid), vortexed immediately and incubated on ice for 10 min, at 100°C for 5 min, and then back on ice for another 5 min. To this mixture 20 µl of *m*-hydroxybiphenyl reagent (0.15% w/v *m*-hydroxybiphenyl in 0.125 M NaOH) was added and vortexed for 1 min. For each sample and standard a negative control was assayed by substituting the *m*-hydroxybiphenyl reagent with 20 µl of 0.125 M NaOH. The absorbance was measured at 520 nm for each sample and standard using the negative controls as blanks. Background uronic acid content from the media was subtracted from samples.

2.10 Analysis of sub-envelope fractions

This section outlines the isolation and analysis of the whole envelope fraction of *P. aeruginosa* strains grown in liquid PI and on solid PIA media (Section 2.2.3).

2.10.1 Cell preparation

For analysis of *P. aeruginosa* strains grown in liquid PI media (Section 2.2.3), a frozen stock was used to inoculate a 5 ml pre-culture, which was incubated overnight. One hundred and fifty microliters of this pre-culture was used to inoculate 150 ml of PI liquid media containing relevant antibiotics (Section 2.3). After incubation for 24 h, cells were harvested by centrifugation (9,000 g at 4ºC for 1 h), washed twice with 1 vol of sterile saline and suspended in 10 ml of 10 mM HEPES buffer pH 7.8.

For analysis of *P. aeruginosa* strains grown on solid PIA media (Section 2.2.3), a frozen stock was used to inoculate a 20 ml pre-culture, which was incubated overnight. Cells from 1 ml of this pre-culture was washed twice with 1 vol of sterile saline, suspended in 200 µl of PI media (Section 2.2.3), and plated on solid PIA media containing relevant antibiotics. After 72 h incubation, biomass from six plates was scrapped off, thoroughly suspended in 150 ml of sterile saline, and cells harvested by centrifugation (9,000 g at 4ºC for 1 h). Cells were washed twice with 150 ml of sterile saline, harvested by centrifugation and then suspended in 10 ml of 10 mM HEPES buffer pH 7.8.

Cells from liquid and solid media suspended in HEPES buffer were either subject to crosslinker treatment (Section 2.10.2) before disruption or disrupted without treatment (Section 2.10.3).

2.10.2 Crosslinker treatment

The homobifunctional crosslinkers discuccinimidyl glutarate (DSG) and dithiobis(succinimidyl propionate) (DSP) have reactive N-hydroxysuccinate ester groups which form irreversible amide bonds with lysine residues (Thermo Scientific Pierce, USA). DSG and DSP have spacer arm lengths of 7.7 and 12 Å, respectively. DSG is non-cleavable while DSP contains a disulphide bond which can be cleaved under reducing conditions. Cell preparations (Section 2.10.1) were washed twice with 1

vol of 10 mM HEPES buffer pH 7.8. Crosslinkers dissolved in DMSO (25 mM) were added to cell suspensions to a final concentration of 1.5 mM and incubated with gentle agitation at 37°C for 30 min (for DSG) or 4°C for 2 h (for DSP). To stop the crosslinking reactions, quenching buffer (1 M Tris-HCl pH 7.8) was added to a final tris concentration of 50 mM followed by incubation at room temperature for 15 min with agitation. After terminating the crosslinking reactions, cells were harvested by centrifugation (15,000 g for 2 min) and washed twice with 10 mM HEPES buffer (pH 7.8) before cell disruption (Section 2.10.3).

2.10.3 Cell disruption

All cell disruption steps were carried out on ice. Cells suspended in 10 mM HEPES buffer from cell preparation (Section 2.10.1) or crosslinking (Section 2.10.2) were supplemented with complete mini-EDTA-free protease inhibitor (Roche, USA) according to the manufacturer's instructions. Cells were disrupted by three passes through a French Press at 4,000 psi.

2.10.4 Preparation of whole membrane fraction

Cells were prepared, crosslinked (where applicable) and disrupted as described in Sections 2.10.1 to 2.10.3. Cellular debris and unlysed cells were removed by centrifugation (9,000 g at 4ºC for 30 min). Next, the whole envelope was harvested from the supernatant by centrifugation (43,000 g at 4ºC for 1 h). The pellet containing the envelope fraction was either stored at -80°C for later analysis, suspended in 10 mM HEPES buffer (pH 7.8) for immediate analysis, or solubilised in binding buffer for pull down experiments via nickel affinity chromatography (Section 2.10.5).

2.10.5 Nickel affinity chromatography

Nickel affinity chromatography was used in pull down experiments under denaturing (using Buffer System A) and non-denaturing (using Buffer System B) conditions to identify potential interaction partners of AlgK. Procedures were carried out on ice and buffers made fresh and kept at 4°C. All buffers in Buffer System A contain 100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, 1% (v/v) Triton X-100, and 0.2% (w/v) sarkosyl at pH 7.8. Buffers A1, A2, A3 and A4 contain 5, 25, 50 and 500 mM imidazole,

respectively. All buffers in Buffer System B contain 100 mM NaH_2PO_4, 10 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100, and 1% (w/v) n-octyl-glucopyranoside at pH 7.8. Buffers B1, B2, B3 and B4 contain 5, 25, 50 and 500 mM imidazole, respectively.

The whole membrane fraction (Section 2.10.4) was prepared and solubilised in 2 ml of binding buffer by rocking on a horizontal shaker at 4°C for 2 h. Binding buffers used in the present study were Buffer A1 (for denaturing conditions) and Buffer B1 (for non-denaturing conditions). The solubilised envelope fractions were subject to nickel affinity chromatography using the Zymo Hisprotein Mini-prep™ kit (Zymo, USA) to purify the His-tagged protein (AlgK-His) with Buffer System A (denaturing) and Buffer System B (non-denaturing), respectively. Three hundred and fifty microliters of affinity gel was used per purification. Four hundred microliters of sample was incubated with the affinity gel for 7 mins, mixed by inversion every 2 mins, and centrifuged (16,000 g for 10 sec). This was repeated several times until all the sample had been mixed with the gel. To remove non-specifically bound proteins, 250 µl of Buffer A2 (or B2 for non-denaturing conditions) was added and centrifuged as above. This was repeated with Buffer A3 (or B3). Finally, the proteins still bound to the affinity gel were eluted in 100 µl of Buffer A4 (or B4 for non-denaturing conditions) after 1 min incubation. The eluted proteins were immediately analysed by SDS-PAGE (2.10.8) or aliquoted, frozen for 1 min in liquid nitrogen, and stored at -80°C for later analysis.

2.10.6 Determination of protein concentration

Protein concentration of samples was determined using a commercial Bradford assay kit (Biorad, USA) based on the assay described by Bradford (1976). Bovine serum albumin at concentrations of 0.80 to 0.05 mg/ml was used to generate a standard curve. Serial two-fold dilutions of samples (up to ×32) were made. Ten microlitres of standards and samples was independently mixed with 200 µl of Bradford reagent (Biorad, USA) in individual wells of a 96 well plate. After incubation at room temperature for 5 min, the absorbance at 595 nm was measured using a plate reader (ELx808iu Ultramicroplate Reader, Bio-Tek instruments, USA).

2.10.7 Sodium dodecyl sulphate gel electrophoresis (SDS- PAGE)

SDS-PAGE as described by Laemmli (1970) was performed to analyse protein samples. 4X Stacking Gel, 4X Separating Gel, and 10X SDS-PAGE Electrophoresis Buffers were prepared. 4X Stacking Gel Buffer contained 81.7 g/L of Tris and 4 g/L of SDS in 1 litre of water, adjusted to pH 8.9. 4X Separating Gel Buffer contained 60.6 g/L Tris and 4 g/L of SDS in 1 litre of water, adjusted to pH 6.8. 10X SDS-PAGE Electrophoresis Buffer contained 30 g/L of Tris, 10 g/L of SDS and 144 g/L of glycine in 1 litre of water, adjusted to pH 8.5. pH adjustments were made with 10 M NaOH and HCl.

The following describes the preparation of two standard 1.5 mm SDS-PAGE gels composed of 8.0 and 3.9% (w/v) acrylamide separating and stacking gels. To prepare 20 ml of 8% (w/v) acrylamide separating gel, 5 ml of 4X Separating Gel Buffer, 5.33 ml of 30% (w/v) acrylamide and 9.67 ml of water were thoroughly mixed before a pinch of Na_2SO_3 was added to degas the mixture. Next, 10 µl of 40% (w/v) APS and 10 µl of TEMED were added to the mixture and stirred for 10 s before the mixture was poured into two 1.5 mm mini Novex® Gel Cassettes (Invitrogen, USA), overlaid with isopropanol, and carefully placed on a level surface for 1 h to allow polymerisation.

Upon polymerisation, the isopropanol was removed and the separating gel thoroughly rinsed with water and dried with lint free wipes. To prepare 5 ml of 3.9% (w/v) acrylamide stacking gel, 1.25 ml of 4X Stacking Gel Buffer, 0.65 ml of 30% (w/v) acrylamide and 3.1 ml of water were mixed thoroughly before a pinch of Na_2SO_3 was added to degas the mixture. Then, 2.5 µl of 40% (w/v) APS and 5 µl of TEMED were added to the mixture and stirred for 10 s before the mixture was poured onto the separating gels and well comb inserted. The stacking gel was left to solidify for 1 h.

SDS-PAGE gels were set up in XCell SureLock™ Mini-Cell Electrophoresis Gel Chambers according to the manufacturer's instructions (Invitrogen Corporation, USA). Gels were submerged in 1X SDS-PAGE Electrophoresis Buffer. Protein samples were prepared (Section 2.10.7.1) and loaded into separate wells. For each gel, a protein molecular weight standard (Section 2.10.7.2) was loaded into its own well. Single 1.5 mm SDS-PAGE gels were run at 15 mA for the first 90-120 min until the proteins had

entered the separating gel. Then, the current was increased to 30 mA for another 90-120 min.

2.10.7.1 Preparation of protein samples for SDS-PAGE

Generally, 1 to 25 µl of protein sample was mixed with 0.2 vol of ×6 SDS-PAGE loading buffer and held at 95°C for 10 min. One to 40 µg of protein was loaded into each well. One millilitre of ×6 SDS-PAGE loading buffer contained 500 µl of 4X Stacking Buffer, 500 µl of glycerol, 120 mg of SDS, 93 mg of DTT, and 0.2 mg of Bromophenol blue.

2.10.7.2 Protein molecular weight standard

A stained protein molecular weight standard, BenchMark^{TM} Pre-stained Protein Ladder (Invitrogen, USA), was used for estimation of protein molecular weight in immunoblot experiments (Section 2.10.8). When using a commercial anti-His antibody (HisProbe-HRP, Thermo Scientific, USA) in immunoblots, a commercial histagged protein molecular weight ladder was employed (6xHis Protein Ladder, Qiagen, USA).

2.10.8 Immunoblot analysis

For the identification of Alg proteins (AlgK, AlgE, Alg44, AlgX) samples were run on SDS-PAGE (Section 2.10.7), transferred to nitrocellulose membrane (Section 2.10.8.1), subject to relevant primary and secondary antibodies, and visualised via chemiluminescence upon exposure and development of X-ray film (Section 2.10.8.2).

2.10.8.1 Transfer to nitrocellulose membrane

Proteins were transferred to nitrocellulose membrane from an SDS-PAGE gel (Section 2.10.7) utilising a semi-dry transfer system (iBlot® Gel Transfer Stacks Nitrocellulose and iBlot® Gel Transfer System, Invitrogen Corporation, USA) according to the manufacturer's instructions using preset program No. 3. The anode (bottom) and cathode (top) consist of copper sheets amalgamated to matrices infused with relevant buffers. The SDS-PAGE gel was carefully placed on the 0.2 µm nitrocellulose membrane which rests on the anode matrix. After removal of air bubbles by using a

roller, a sheet of filter paper moistened with de-ionised water followed by the cathode was placed on the top of the gel. The sponge designed to ensure application of even pressure was placed on the top of the cathode, the lid securely fastened, and the transfer programme initiated.

2.10.8.2 Blocking, antibody application, and visualisation

After transfer, the nitrocellulose membrane (Section 2.10.8.1) was washed thrice with Tris buffered saline supplemented with Tween 20 (TBST: 150 mM NaCl, 10 mM Tris-HCl and 0.1% (v/v) Tween 20 at pH 7.8) for 5 min and blocked overnight in TBST supplemented with 3% (w/v) skim milk at 4°C. The membrane was washed thrice and incubated for 1 h with rabbit anti-Alg antibody of interest at a 1:10,000 (v/v) ratio in TBST supplemented with 2% (w/v) skim milk. Following primary antibody incubation, the membrane was washed thrice with TBST and incubated with goat anti-rabbit antibody conjugated to horse radish peroxidase (Abcam, England) at a 1:5,000 (v/v) ratio in TBST supplemented with 2% (w/v) skim milk. After another three washes with TBST, the membrane was incubated with 5 ml of Super Signal West Pico Stable Peroxide and 5 ml of Luminol/Enhancer solutions (Thermo Scientific, USA) for 5 min. All incubation and wash steps were carried out with gentle agitation. Incubations with light sensitive antibodies and substrates were carried out in the dark. For visualisation of bands, the membranes were exposed to BioMax XAR film (Kodak, USA) and images were developed using a developer (Kodak X-Omat-100, USA).

For detection of histagged proteins a commercial anti-His antibody conjugated to horse radish peroxidase (HisProbe-HRP Kit, Thermo Scientific, USA) was employed according to the manufacturer's instructions. Briefly, proteins were transferred to the nitrocellulose membrane as described above. Then the membrane was blocked overnight in TBST supplemented with 1% (w/v) BSA. After washing the membrane twice in 15 ml of TBST, it was incubated with the antibody at a 1:5,000 (v/v) ratio in TBST for 1 h at room temperature in the dark. Following another 4 washes with TBST, the membrane was incubated with the substrate and bands of interest were visualised as described above.

CHAPTER THREE

RESULTS

3.1 Molecular cloning

To approach the aim of this study (Section 1.7) an isogenic marker free *algK* deletion mutant was generated from the alginate over-producer *P. aeruginosa* PDO300 (Section 3.1.1). This mutant was complemented with the *algK* gene or *algK-his* (encoding an AlgK protein with a C-terminal hexahistidine tag) *in trans* and *in cis*, generating four complemented strains (Section 3.1.2).

The PDO300Δ*algE*, Δ*alg44* and Δ*algX* mutants used in this study were previously generated by Hay et al. (2010b), Remminghorst & Rehm (2006a) and Gutsche et al. (2006), respectively. The *in cis* complemented strains PDO300Δ*algE*(CTXP*alg*:*algE*) Δ*alg44*(CTXP*alg*:*alg44*), and PDO300Δ*algX*(CTXP*alg*:*algX*) used in the present study were produced by Rehman et al. (2013).

3.1.1 Generation of isogenic marker free PDO300Δ*algK* mutant

Figure 12 outlines the strategy for generating an isogenic marker free Δ*algK* deletion mutant from *P. aeruginosa* PDO300.

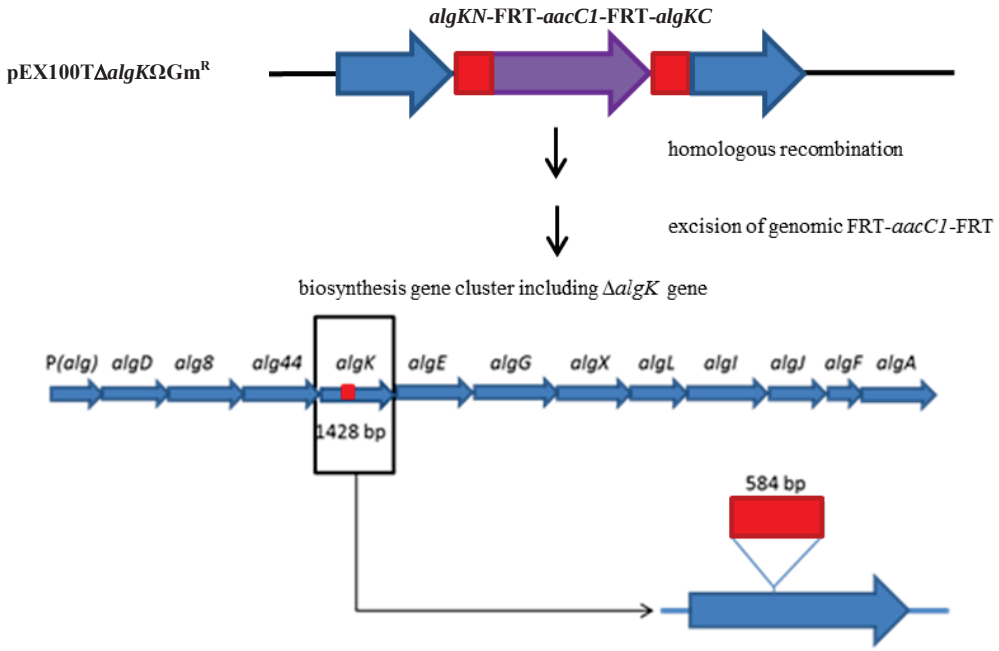

Figure 12. Strategy for generation of *algK* mutant. The plasmid pEX100TΔ*algK*ΩGm^R which carried a cassette, *algKN*-FRT-*aacC1*-FRT-*algKC*, designed to disrupt the *algK* gene was constructed as described in Section 3.1.1.1. This cassette is composed of an N-terminal (*algKN*) and a C-terminal (*algKC)* region of the *algK* ORF (large short blue arrows) sitting on alternate sides of a gentamycin resistance gene, *aacC1* (purple arrow), which is flanked by FRT recognition sites (red squares). The plasmid was introduced into *P. aeruginosa* PDO300 and a homologous recombination event was selected for as outlined in Section 3.1.1.2, whereby, the middle region of the *algK* gene (red rectangle, 584 bp from 461-1044 bp of the *algK* ORF) was replaced with the FRT-*aacC1*-FRT marker, generating the mutant PDO300Δ*algK*ΩGm^R. The marker was subsequently excised by introducing the flip recombinase encoding plasmid pFLP2 and after removing this plasmid the isogenic marker free mutant PDO300Δ*algK* was created (Section 3.1.1.3).

3.1.1.1 Construction of plasmid pEX100TΔ*algK*ΩGm^R

Figure 13 illustrates the strategy for constructing the plasmid pEX100TΔ*algK*ΩGm^R. To make this plasmid, several DNA fragments (*algKN*, *algKC*, FRT-*aacC1*-FRT, and linear pEX100T) were prepared. The first two fragments, *algKN* (460 bp) and *algKC* (384 bp) which comprise two regions (1 to 460 bp and 1,045 to 1,428 bp) of the *algK* coding region, were obtained by cloning using pGEMT®-Easy Vector System (Section 2.7.8). The fragments *algKN* and *algKC* were acquired by PCR (Section 2.7.6) of chromosomal DNA isolated from *P. aeruginosa* PDO300 (Section 2.7.1) using *Taq*

polymerase and primer pairs *algK*NF(*Eco*RV) and *algK*NR(*Bam*HI), and *algK*CF(*Bam*HI) and *algK*CR(*Eco*RV). The PCR products were gel purified (Section 2.7.3), ligated into pGEMt-Easy (Section 2.7.8.2), and propagated in *E. coli* TOP10 on X-Gal media (Section 2.2.2) containing ampicillin (Section 2.3).

Plasmid DNA was isolated (Section 2.7.2), screened by restriction analysis (Section 2.7.8.3), and confirmed by DNA sequencing (Section 2.7.10). The resulting plasmids, pGEMt-*algKN* and pGEMt-*algKC*, were hydrolysed by *Eco*RV, gel purified (Section 2.7.3), A-tailed (Section 2.7.8.1), and digested with *Bam*HI, generating A-tailed and *Bam*HI sticky-ended *algKN* and *algKC* fragments. These fragments were ligated into pGEMt-Easy (Section 2.7.8.2) with the FRT-*aacC1*-FRT fragment (~1,100 bp) attained by *Bam*HI hydrolysis of plasmid pPS856. The resulting plasmid pGEMt-*algKNC*ΩGm was propagated in *E. coli* TOP10 on X-Gal media (Section 2.2.2) containing gentamycin (Section 2.3). The sequence-confirmed pGEMt-*algKNC*ΩGm plasmid was used as a template for amplifying the *algKN*-FRT-*aacC1*-FRT-*algKC* cassette by PCR with *Pfx* polymerase in reaction mixture (B) and primers *algK*NF(*Eco*RV) and *algK*CR(*Eco*RV) (Section 2.7.6). The PCR product was ligated (Section 2.7.9) into the *Sma*I site of the suicide vector pEX100T, generating pEX100TΔ*algK*ΩGm^R which was proliferated in *E. coli* TOP10 on LB (Section 2.2.1) supplemented with gentamycin (Section 2.3). Plasmid DNA (Section 2.7.2) was screened by restriction digestion with *Bam*HI and *Eco*RV (Section 2.7.7).

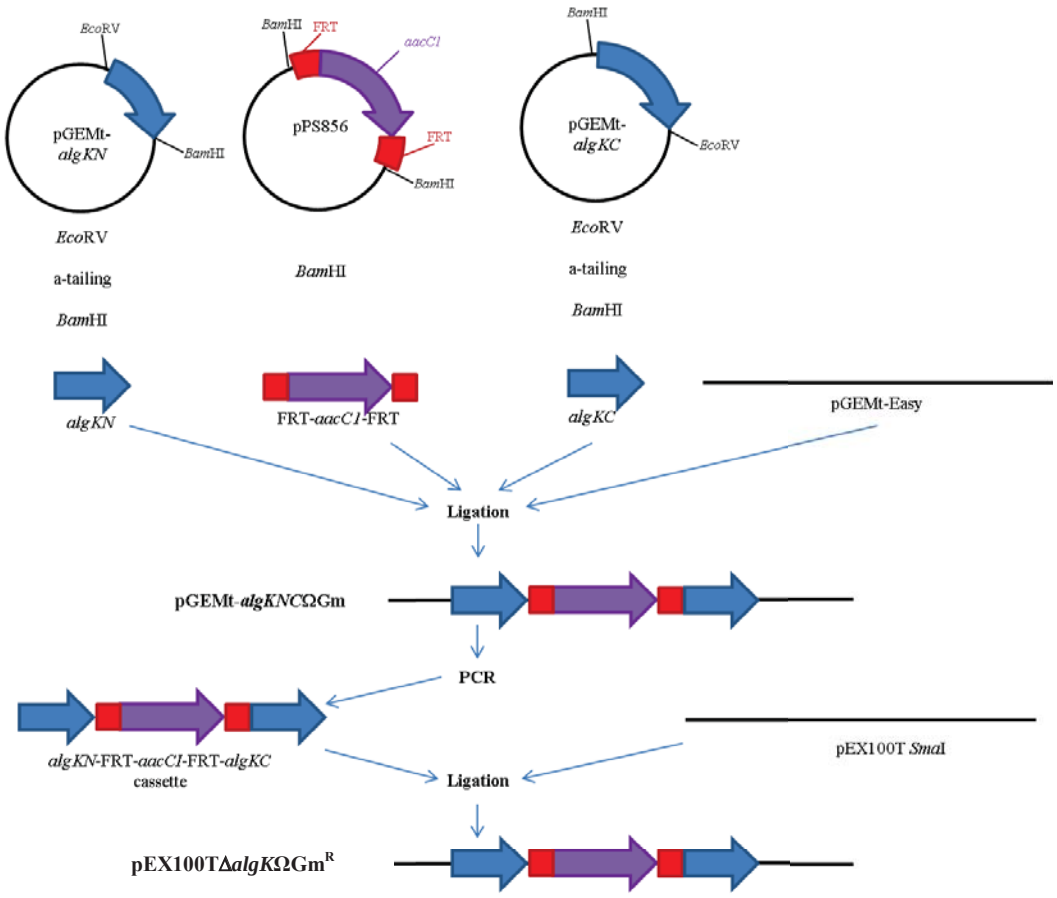

Figure 13. Construction of plasmid pEX100TΔ*algK*ΩGm^{R}. The confirmed plasmids pGEMt-*algKN* and pGEMt-*algKC* were hydrolysed with *Eco*RV, A-tailed, digested with *Bam*HI and ligated into pGEMt-Easy with a ~1,100 bp *Bam*HI fragment (FRT- *aacC1*-FRT) from plasmid pPS856. The resulting plasmid, pGEMt-*algKNC*ΩGm, was used as a template to obtain the *algKN*-FRT-*aacC1*-FRT-*algKC* cassette by PCR using *Pfx* polymerase and primers *algK*NF(EcoRV) and *algK*CR(EcoRV). The PCR product was ligated with a *Sma*I hydrolysed pEX100T vector, yielding the plasmid, pEX100TΔ*algK*ΩGm^{R}, which was propagated in *E. coli* TOP10 on LB gm. This plasmid was confirmed by independent restriction endonuclease analysis with *Bam*HI and *Eco*RV.

3.1.1.2 Generation of PDO300Δ*algK*ΩGm^R

The confirmed plasmid, pEX100TΔ*algK*ΩGm^R, was transconjugated into *P. aeruginosa* PDO300 (Section 2.6.3). Transconjugants were selected for on MSM (Section 2.2.5) containing gentamycin (Section 2.3) and 5% (w/v) sucrose. Gentamycin selects for the gentamycin resistance marker, the *aacC1* gene, within the cassette (*algKN*-FRT-*accC1*-FRT-*algKC*) designed to disrupt the *algK* gene while sucrose selects against the *sacB* gene on the backbone of the plasmid. Under these conditions, a double cross over event was selected for, creating the strain PDO300Δ*algK*ΩGm which was verified by gentamycin resistance and PCR (yielding a ~2,000 bp PCR product) using the primers *algK*(upXout) and *algK*(downXout) which immediately flanked the *algK* ORF in the chromosome (Figure 14a, Lane 4).

3.1.1.3 Generation of PDO300Δ*algK*

To generate an isogenic marker free Δ*algK* deletion mutant from the strain PDO300Δ*algK*ΩGm^R, the gentamycin resistance marker (FRT-*aacC1*-FRT) was removed by introducing the flip recombinase encoding plasmid, pFLP2 (Kovach et al. 1995), into PDO300Δ*algK*ΩGm^R using the donor strain *E. coli* SM10 (Section 2.6.3). Successful transconjugants were selected for on PIA (Section 2.2.3) containing carbenicillin (Section 2.3) for 24 h. Excision of the marker was confirmed by sensitivity to gentamycin as well as PCR (yielding a ~900 bp product) using primers *algK*(upXout) and *algK*(downXout) (Figure 14a, Lane 5). To remove the pFLP2 plasmid which also carried a *sacB* gene, colonies were transferred to PIA (Section 2.2.3.1) containing 5% (w/v) sucrose. The elimination of pFLP2 was validated by sensitivity to carbenicillin.

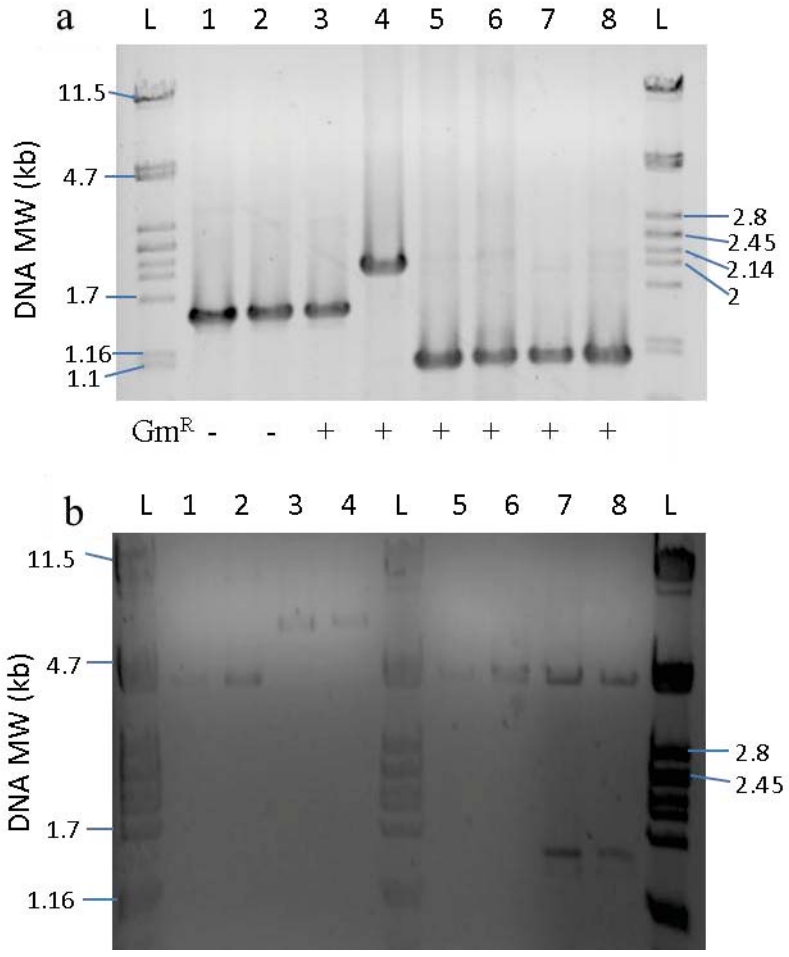

Figure 14. Confirmation of *algK* deletion mutant and strains harbouring pBBR1MCS-5 derived plasmids. (a) Colony PCR using primers *algK*(upXout) and *algK*(downXout) which immediately flank the *algK* ORF in the genomic DNA: Lane 1 = PDO300 genomic DNA, Lane 2 = PDO300, Lane 3 = PDO300(MCS-5) and Lane 4 = PDO300Δ*algK*ΩGm; Lanes 5-8 represent PDO300Δ*algK* strains transformed with: no plasmid (Lane 5), MCS-5 (Lane 6), MCS-5:*algK* (Lane 7), and MCS-5:*algK-his* (Lane 8). Gm^R is resistance/sensitivity (+/-) to gentamycin. (b) *Bam*HI and *Hind*III restriction endonuclease digestion of plasmids isolated from various strains. Lanes 1-4 represent uncut plasmids from PDO300(MCS-5) (Lane 1), Δ*algK*(MCS-5) (Lane 2), Δ*algK*(MCS-5::*algK*) (Lane 3), and Δ*algK*(MCS-5:*algK-his*) (Lane 4). Lanes 5-8 represent plasmids from Lanes 1-4 digested with *Bam*HI and *Hind*III. Lanes labelled L contain DNA molecular weight markers. MCS-5 is pBBR1MCS-5. The sizes of MCS-5, *algK*/*algK-his* and *algK*ΩGm are ~3.5 kb, ~1.5 kb/~1.5 kb and ~2 kb, respectively.

3.1.2 Complementation of *algK* mutant

For complementation, the *algK* ORF and *algK-his* were independently introduced into the *algK* mutant *in trans* (on a plasmid) and *in cis* (via chromosomal integration) (Figure 15). These complementation strategies offered the opportunity to examine the effect of *algK* gene dosage/copy number on various phenotypes (Section 3.2).

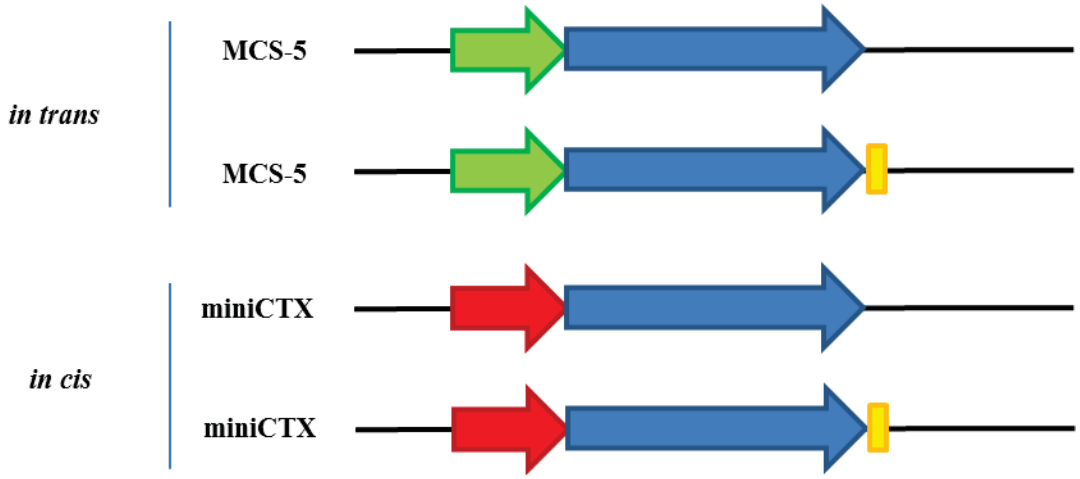

Figure 15. Four approaches to complementing *algK* mutant. The first two approaches involved *in trans* complementation where the *algK* gene (blue arrow) or *algK* gene with a hexahistidine tag (blue arrow + yellow box = *algK-his*) was introduced independently into the *algK* mutant under the control of the constitutive *lacZ* promoter (green arrow) on the medium copy number plasmids pBBR1MCS-5:*algK* and pBBR1MCS-5:*algK-his*. The third and fourth approaches involved *in cis* complementation where the *algK* gene or *algK-his* was integrated into the chromosome of the *algK* mutant at the *attB* site under the control of the *algD* promoter (red arrow) utilising the plasmids miniCTXP*alg*:*algK* and miniCTXP*alg*:*algK-his*. MCS-5 = pBBR1MCS-5 and miniCTX = miniCTX-2.

3.1.2.1 Preparation of fragments for plasmid construction

For *in trans* complementation the plasmids pBBR1MCS-5:*algK* and pBBR1MCS-5:*algK-his* were constructed (Figure 16). For *in cis* complementation the plasmids miniCTXP*alg*:*algK* and miniCTXP*alg*:*algK-his* were produced (Figure 17). To generate these plasmids, DNA fragments (*algK*, *algK-his,* and P*alg*) were prepared using the pGEMT®-Easy Vector System according to the general strategy outlined in Section 2.7.8 (Figure 11). The fragments *algK*, *algK-his*, and *Palg* were acquired by *Pfx*

polymerase using chromosomal DNA isolated from *P. aeruginosa* PDO300 as a template (Section 2.7.1) in reaction mixture (A) (Section 2.7.6). The primer pairs used to obtain *algK*, *algK-his*, and *Palg* were *algK*N(HiSDNd) and *algK*C(*Bam*HI), *algK*N(HiSDNd) and *algK*C(6xhis*Bam*HI), and PstAlgPF and HiIIIAlgPR, respectively. All three PCR products were A-tailed (Section 2.7.8.1), independently ligated into pGEMt-Easy (Section 2.7.8.2), and propagated in *E. coli* TOP10 on X-Gal medium (Section 2.2.2) supplemented with ampicillin (Section 2.3). The resulting plasmids pGEMt-*algK*, pGEMt-*algK-his* and pGEMt-P*alg* were screened (Section 2.7.8.3) and verified by DNA sequencing (Section 2.7.10).

The plasmids pGEMt-*algK* and pGEMt-*algK-his* were digested by *Bam*HI and *Hind*III, releasing the fragments *algK* and *algK-his* (Figures 16 & 17). The plasmid pGEMt-P*alg* was hydrolysed with *Pst*I and *Hind*III, liberating the P*alg* fragment (Figure 17). These fragments (*algK*, *algK-his*, and P*alg*) were gel purified (Section 2.7.3) and quantified (Section 2.7.4). The relevant plasmids used for *in trans* and *in cis* complementation (pBBR1MCS-5 and miniCTX-2, respectively) were linearised in separate restriction endonuclease digestions using *Hind*III and *Bam*HI, and *Pst*I and *Bam*HI, respectively (Figures 16 & 17). The linearised plasmids were gel purified (Section 2.7.3) and quantified (Section 2.7.4), ready for subsequent ligation and cloning.

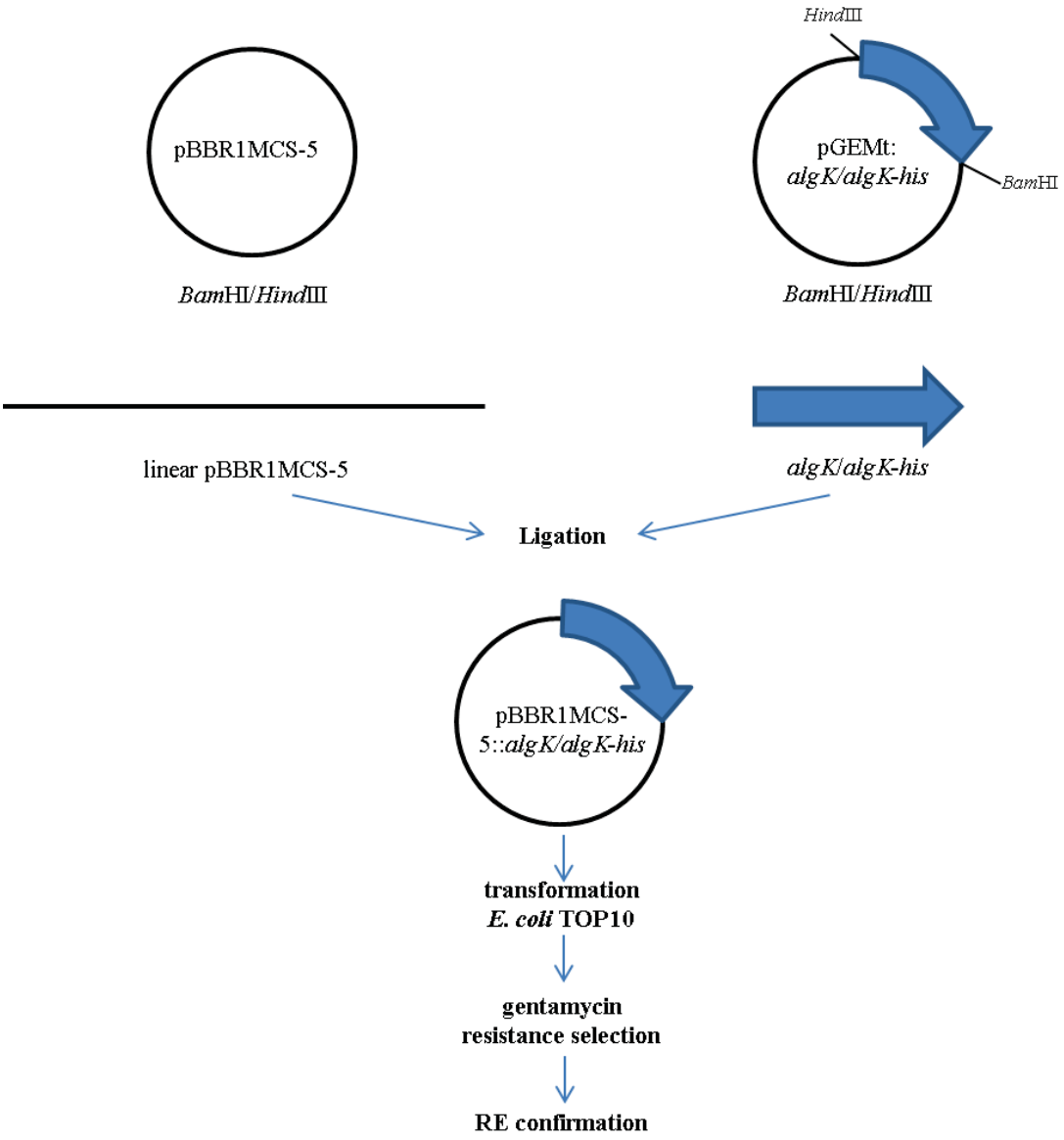

Figure 16. Construction of plasmids for *in trans* complementation. The confirmed plasmids pGEMt-*algK* and pGEMt-*algK-his* (referred to as pGEMt-*algK*/*algK-his*) were hydrolysed with *Bam*HI and *Hind*III. The *algK* and *algK-his* fragments were independently ligated into the corresponding sites of pBBR1MCS-5, producing the plasmids pBBR1MCS-5:*algK* and pBBR1MCS-5:*algK-his* which were transformed into *E. coli* TOP10 and plated on LB gm. The plasmids of interest were confirmed by *Bam*HI and *Hind*III digest.

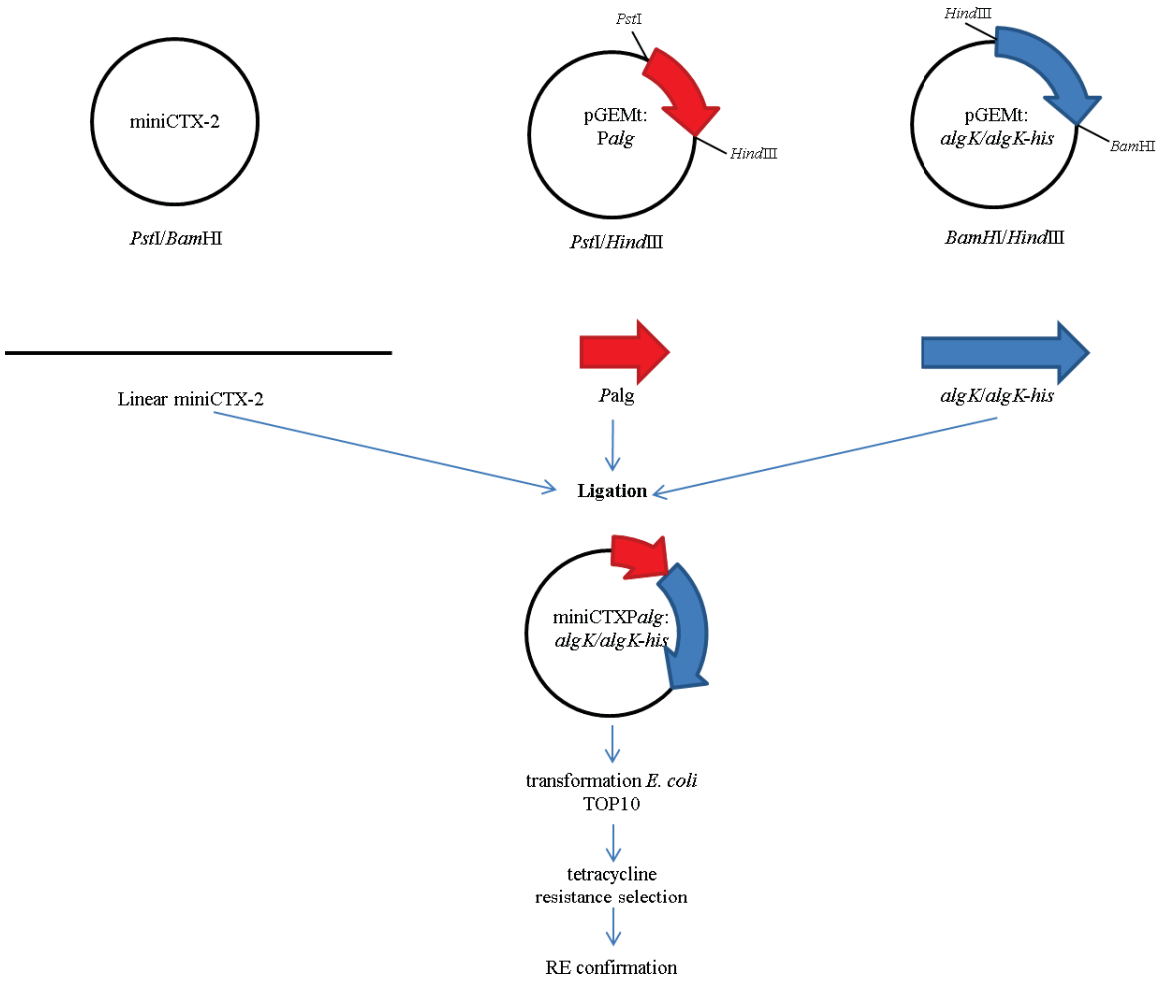

Figure 17. Construction of plasmids for *in cis* complementation. The miniCTX-2 vector was linearised by *Pst*I and *Bam*HI hydrolysis. The confirmed plasmid pGEMt-P*alg* was digested with *Pst*I and *Hind*III, liberating the *Palg* fragment. The sequence verified plasmids pGEMt-*algK* and pGEMt-*algK-his* (referred to as pGEMt:*algK*/*algK-his*) were hydrolysed with *Bam*HI and *Hind*III, releasing the *algK* and *algK-his* fragments. To construct the plasmid miniCTXP*alg*:*algK* for *in cis* complementation, the linearised vector was ligated with the *Palg* and *algK* fragments. To generate the plasmid miniCTXP*alg*:*algK-his* the vector was ligated with the *Palg* and *algK-his* fragments. These plasmids were transformed into *E. coli* TOP10 and plated on LB tet. The final plasmids were verified by *Pst*I, *Bam*HI and *Hind*III restriction analysis.

3.1.2.2 Construction and confirmation of plasmids and complemented strains

The fragments *algK* and *algK-his* generated in Section 3.1.2.1 were separately ligated into the linearised pBBR1MCS-5, yielding the plasmids pBBR1MCS-5:*algK* and pBBR1MCS-5:*algK-his* which were used for *in trans* complementation (Figure 16). These plasmids were confirmed by restriction analysis with *Bam*HI and *Hind*III, and then electroporated (Section 2.6.2) into PDO300Δ*algK*, generating the *in trans* complemented strains Δ*algK*(MCS-5:*algK*) and Δ*algK*(MCS-5:*algK*-his). These strains were selected for on PIA medium (Section 2.2.3.1) containing gentamycin (Section 2.3). The empty vector pBBR1MCS-5 was also electroporated into PDO300 and PDO300Δ*algK,* creating the control strains PDO300(MCS-5) and Δ*algK*(MCS-5). The strains PDO300(MCS-5), Δ*algK*(MCS-5), ΔalgK(MCS-5:*algK*) and Δ*algK*(MCS-5:*algK*-his) were verified by PCR (Section 2.7.6) using primers *algK*(upXout) and *algK*(downXout), and restriction analysis of plasmids purified from each strain using *Bam*HI and *Hind*III (Figure 14b).

To construct the plasmids miniCTXPalg:*algK* and miniCTXPalg:*algK-his* for *in cis* complementation, the *algK* and *algK-his* fragments prepared in Section 3.1.2.1 were separately ligated with the P*alg* fragment (Section 3.1.2.1) and linearised miniCTX-2 vector (Figure 17). These plasmids were independently transconjugated (Section 2.6.3) into PDO300Δ*algK* using *E. coli* S17-1. Successful transconjugants were selected for on PIA media (Section 2.2.3) supplemented with tetracycline (Section 2.3). Successful integration of these plasmids at the *attB* site was confirmed by PCR (Section 2.7.6) using Pser Up and Pser Down primers which flank this region of the chromosome (Hoang et al. 2000) (Figure 18). The miniCTX-2 backbone including the tetracycline resistance marker was removed by introducing the plasmid pFLP2 (Kovach et al. 1995) which was later removed by counter selection on MSM medium (Section 2.2.5) containing 5% (w/v) sucrose. Excision of miniCTX-2 backbone, producing the strains Δ*algK*(CTXP*alg:algK*) and Δ*algK*(CTXP*alg:algK-his*), was substantiated by sensitivity to tetracycline as well as PCR (Section 2.7.6) using Pser Up and Pser Down primers (Figure 18). Elimination of the pFLP2 plasmid was confirmed by sensitivity to

carbenicillin. The control strains, PDO300Δ*algK*(CTX) and PDO300(CTX), were also generated.

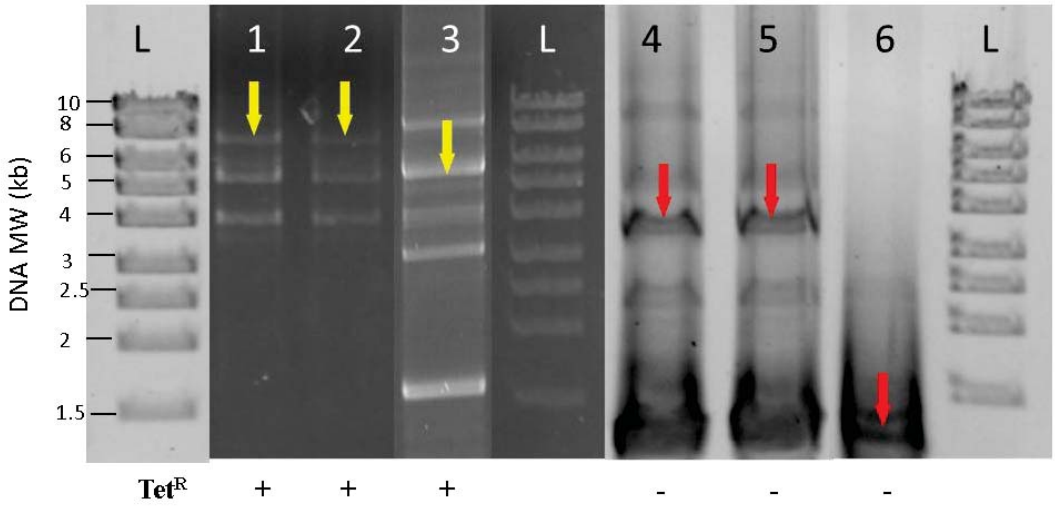

Figure 18. Confirmation of strains complemented *in cis*. Colony PCR using primers Pser Up and Pser Down which flank the *attB* site in the genomic DNA: Lanes 1-3 represent strains with miniCTX-2 derived plasmids chromosomally integrated at the *attB* site and resistant (+) to tetracycline. Lane 1 = Δ*algK*(miniCTXPalg:*algK*), lane 2 = Δ*algK*(miniCTXPalg:*algK-his*) and lane 3 = Δ*algK*(miniCTX). From left to right, DNA bands marked by yellow arrows have molecular weights of ~7.5, 7.5 and 5 kb. Lanes 4-6 represent strains where the backbone of miniCTX-2 derived plasmids was excised and susceptible (-) to tetracycline. Lane 4 = Δ*algK*(CTXPalg:*algK*), lane 5 = Δ*algK*(CTXPalg:*algK-his*) and lane 6 = Δ*algK*(CTX). From left to right, DNA bands of interest marked by red arrows have molecular weights of ~3.5, 3.5 and 1.3 kb.

3.2 Phenotypic characterisation of strains generated

The effect of deleting and replacing the *algK* gene on growth rate and colony morphology (Section 3.2.1) and alginate production (Section 3.2.2) was assessed. The sub-cellular localisation of AlgK (Section 3.2.3) and the effect of AlgK absence on the stability of other subunits and effect of the absence of other subunits on the stability of AlgK (Section 3.2.4) in the envelope fraction were also investigated. Based on mutual stability results, putative protein-protein interactions were proposed and attempts were made through pull down experiments to verify potential interactions of AlgK (Section 3.2.5).

3.2.1 Growth rate and colony morphology

Here we examined the effect of the deletion of *algK* and subsequent *in cis* complementation on growth rate to: (i) assess whether the Δ*algK* mutant produced alginate, and (ii) to establish how long it took to reach mid stationary phase to allow us to harvest samples at a time when alginate production is most pronounced. When cultivated in liquid media, the Δ*algK* mutant displayed a faster growth rate and reached a greater optical density compared to PDO300 (Figure 19). Expressing the *algK* gene *in cis* was unable to restore growth rate or maximum optical density to PDO300 levels (Figure 19).

In this section, we also assessed the effect of deletion of *algK* on colony morphology to (i) determine whether *algK* was required for alginate production (an alginate over-producer would display a mucoid colony morphology) and (ii) to explore whether the disruption of the *algK* gene altered other colony phenotypes such as pigmentation and morphology. Photographs of colonies from each strain are shown in Figure 20. Colonies of PDO300, PDO300(MCS-5) and PDO300(CTX) were completely mucoid (Figure 20, row 1) while the Δ*algK* mutants PDO300Δ*algK*, PDO300Δ*algK*(MCS-5) and PDO300Δ*algK*(CTX) were entirely non-mucoid (Figure 20, row 2). Introducing either the *algK* gene or the *algK* gene C-terminally fused to a hexahistidine tag (*algK-his*) *in trans* or *in cis* yielded colonies with a mucoid centre and non-mucoid periphery (Figure 20, rows 3 & 4). Interestingly, the complemented strains took longer (36 h incubation at 37°C) compared to the wild type strains (24 h) to become mucoid. Variable levels of

pigmentation were observed; however, no correlation with strain identity was found. Colony shape and size were similar between the strains.

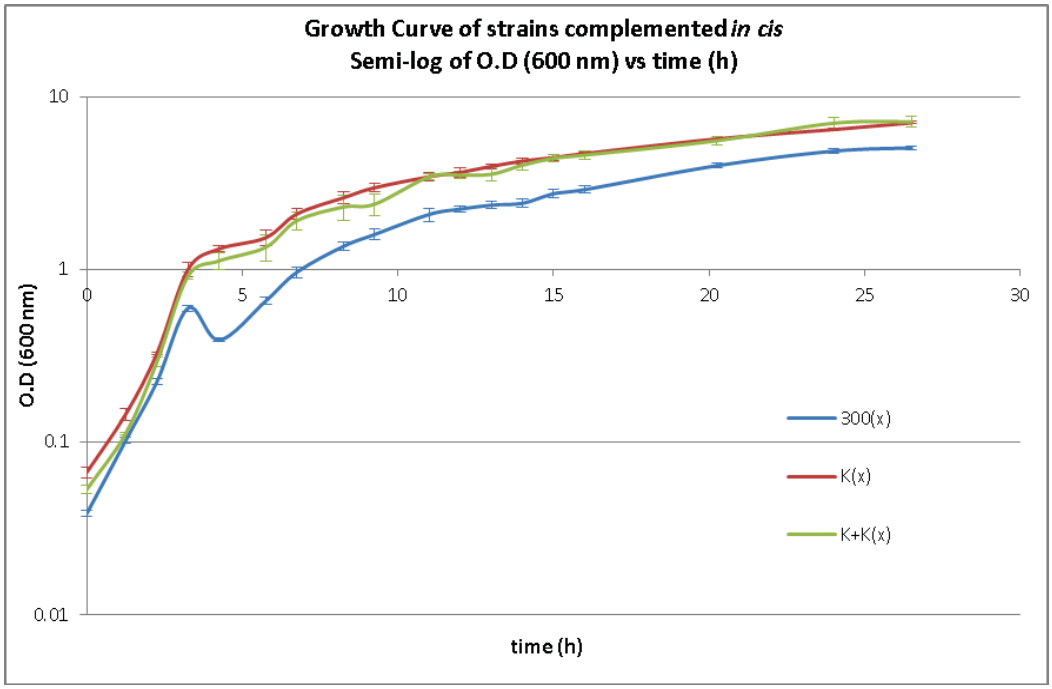

Figure 19. Growth curve of strains complemented *in cis*. Strains were inoculated from overnight culture into 50 ml PI media to an initial optical density at 600 nm (OD_{600nm}) of 0.05 and cultivated at 37°C with shaking (200 rpm). The $OD_{600\ nm}$ was measured hourly for the first 16 hrs, then every 3 to 5 hrs until 27 h was reached. Error bars represent standard deviation from the mean value of three independent experiments. 300(x) = *P. aeruginosa* PDO300(CTX), K(x) = PDO300Δ*algK*(CTX), and K+K(x) = PDO300Δ*algK*(CTXP*alg*:*algK*).

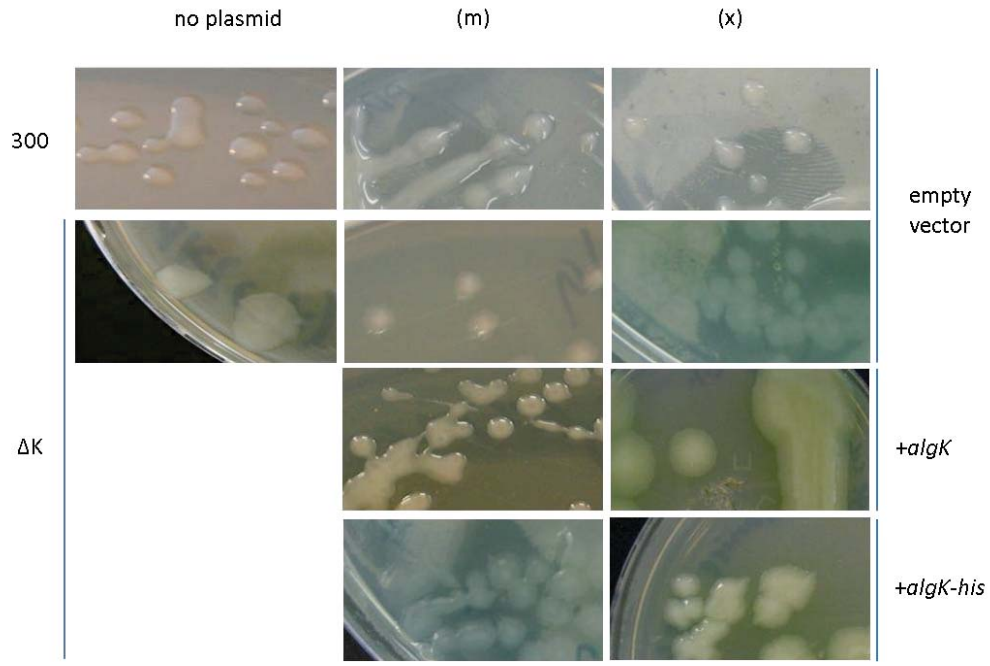

Figure 20. Colony morphology of representative colonies of generated strains. Row 1 left to right: PDO300, PDO300(MCS-5) and PDO300(CTX); row 2 left to right: PDO300Δ*algK*, Δ*algK*(MCS-5) and Δ*algK*(CTX); row 3 left to right: Δ*algK*(MCS-5::*algK*) and Δ*algK*(CTXP*alg*:*algK*), and row 4 left to right: Δ*algK*(MCS-5::*algK-his*) and Δ*algK*(CTXP*alg*:*algK-his*). 300 = PDO300; ΔK = PDO300Δ*algK*; no plasmid = strains with no plasmid; (m) = strains with pBBR1MCS-5 derived plasmids; (x) = strains with miniCTX-2 derived plasmids with backbone removed; +*algK* = strains complemented with *algK* ORF, and +*algK-his* = strains complemented with *algK-his*.

3.2.2 Characterisation of alginate production

To investigate the requirement of *algK* for alginate production, we quantified the alginate yield of strains grown on solid media (Sections 2.9.1 and 2.9.3) (Figure 21). The wild type strains PDO300(CTX) and PDO300(MCS-5) produced 0.559 ± 0.129 (mean ± SD) and 2.770 ± 0.578 g Alg/g CDW (grams of alginate per gram of cellular dry weight), respectively. In contrast, the Δ*algK* mutants PDO300Δ*algK*(CTX) and PDO300Δ*algK*(MCS-5) did not produce alginate. Introducing the *algK* gene or *algK-his* into the Δ*algK* mutant *in trans* or *in cis* restored alginate production, indicating that the disruption of the *algK* gene did not have polar effects on downstream genes.

The Δ*algK* mutants harbouring empty vectors [Δ*algK*(MCS-5) and Δ*algK*(CTX)] did not produce high molecular weight alginate, indicating that *algK* is essential for alginate biosynthesis. However, this result could not distinguish whether *algK* functioned during polymerisation or translocation/secretion. Previous studies conducted in liquid media have shown that deletion of genes involved in polymerisation (*alg8* and *alg44*) abolished alginate and free uronic acid oligomer production while deletion of genes involved in translocation/secretion (*algG, algX*, *algE*) released high quantities of free uronic acid oligomers, which are alginate degradation products generated by the alginate lyase AlgL (Jain et al. 2003, Robles-Price et al. 2004, Oglesby et al. 2008, Hay et al. 2010).

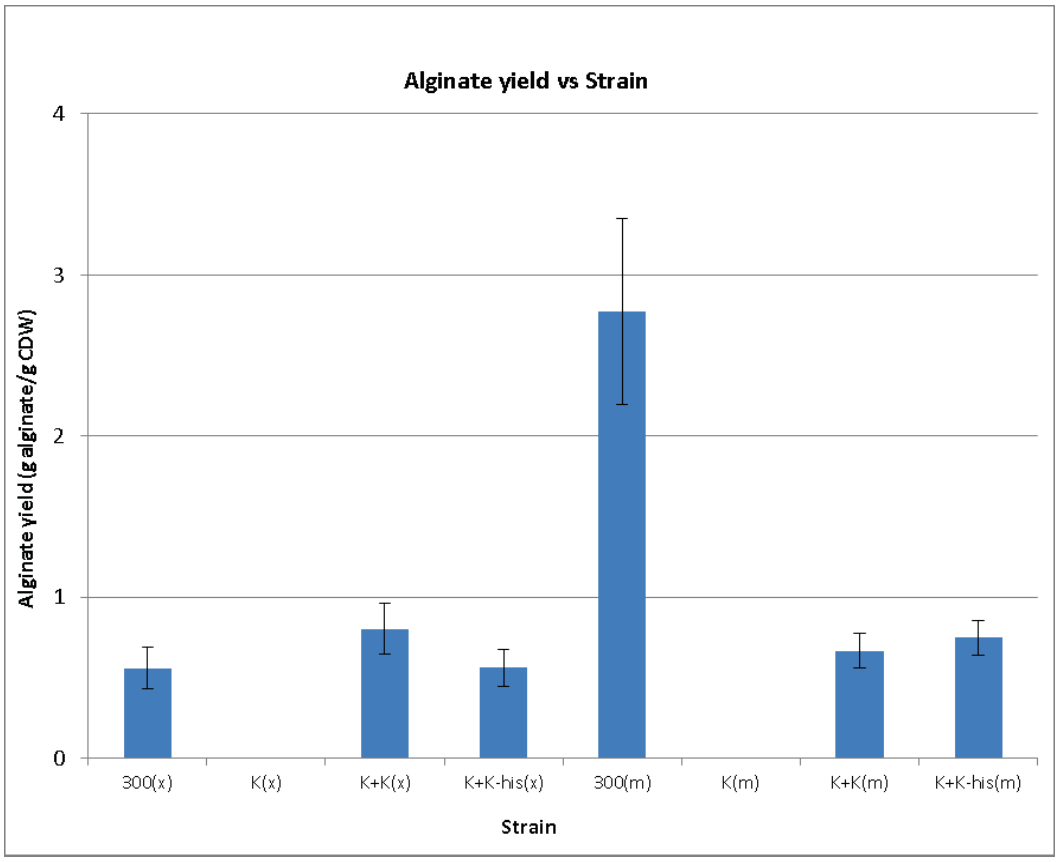

Figure 21. High molecular weight alginate production of generated strains. Strains were grown on PIA media with relevant antibiotics at 37°C for 72 h. Alginate samples were prepared and uronic acid content determine as described in Sections 2.9.1 and 2.9.3. Values of alginate yield are standardised to cellular dry weight. Displayed values are averages and error bars represent standard deviation of three independent experiments. 300(x) = *P. aeruginosa* PDO300(CTX), K(x) = Δ*algK*(CTX), K+K(x) = Δ*algK*(CTXP*alg*:*algK*), K+K-his(x) = ΔalgK(CTXP*alg*:*algK-his*), 300(m) = *P. aeruginosa* PDO300(MCS-5), K(m) = Δ*algK*(MCS-5), K+K(m) = Δ*algK*(MCS-5:*algK*), and K+K-his(m) = Δ*algK*(MCS-5:*algK-his*).

To determine whether *algK* functioned at the level of polymerisation or translocation/secretion, we measured the uronic acid size distribution of the culture supernatants of several strains. The strains PDO300 and Δ*algK* mutant strains harbouring empty vectors as well as the complemented strains were cultivated in liquid media to late logarithmic growth phase (Section 2.9.2) and the uronic acid content of (i) the culture supernatant and (ii) culture supernatant after filtration (10 kDa cut off) was determined (Figure 22). The uronic acid content of (i) corresponded to the total uronic acid content including high molecular weight alginate and alginate degradation products while the uronic acid content of (ii) equated to just the alginate degradation products. If AlgK was essential for polymerisation, then no uronic acids should be present in samples (i) and (ii). However, if *algK* participated in translocation/secretion, equally high quantities of uronic acid should be detected in both samples.

The culture supernatants of the wild type strains PDO300(CTX) and PDO300(MCS-5) contained 0.2254 ± 0.0235 (mean ± SD) and 0.5132 ± 0.0279 g UA/g CDW (grams of uronic acid per gram of cellular dry weight) with approximately a quarter of this being smaller than 10 kDa, indicating that a proportion of alginate was degraded during normal production (Figure 22). Upon deletion of the *algK* gene, the uronic acid content of the culture supernatant was reduced by twenty folds with 100% of uronic acid being smaller than 10 kDa (Figure 22). Introducing the *algK* ORF and *algK-his in trans* or *in cis* into the Δ*algK* mutant did not return the uronic acid content of culture supernatants to wild type levels (Figure 22). However, a substantial proportion of uronic acid in the culture supernatant of the complemented strains was above 10 kDa, suggesting that some function was restored (Figure 29).

Previous studies by Remminghorst & Rehm (2006a) and Gutsche et al. (2006) have proposed that Alg44 and AlgX are required for polymerisation. In the present study, the extremely low uronic acid content of the Δ*algK* mutant was comparable to that of Δ*alg44* and Δ*algX* mutants, suggesting that Alg44, AlgX and AlgK are required for polymerisation (Figure 23). By comparison, the Δ*algE* mutant yielded high quantities of uronic acid – slightly more than the wild type – in its culture supernatant with all of it

being smaller than 10 kDa, signifying that polymerisation was not affected in this strain (Figure 23).

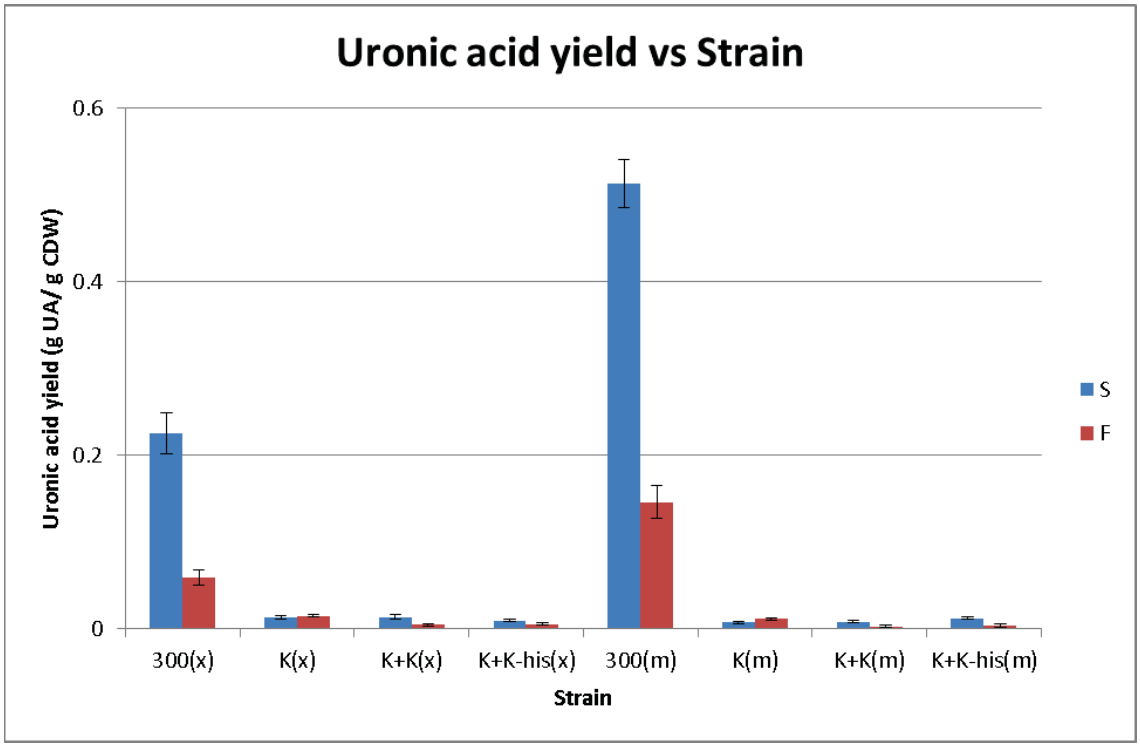

Figure 22. Determination of uronic acid concentration in unfiltered and filtered culture supernatants of PDO300, Δ*algK* mutant and its complemented strains. Strains were inoculated from overnight cultures into 50 ml PI media with relevant antibiotics to an initial O.D $_{600nm}$ of 0.05 and cultivated at 37°C for 24 h. Uronic acid content of unfiltered culture supernatants (S) and filtered supernatants (F) (at 10 kDa cut off) was determined as described in Sections 2.9.2 and 2.9.3. Background uronic acid of growth media was determined and subtracted from values. Values of uronic acid yield were standardised to cellular dry weight. Displayed values are means and error bars represent standard deviations from three independent experiments. 300(x) = *P. aeruginosa* PDO300(CTX), K(x) = Δ*algK*(CTX), K+K(x) = Δ*algK*(CTXP*alg*:*algK*), K+K-his(x) = ΔalgK(CTXP*alg*:*algK-his*), 300(m) = *P. aeruginosa* PDO300(MCS-5), K(m) = Δ*algK*(MCS-5), K+K(m) = Δ*algK*(MCS-5:*algK*), and K+K-his(m) = Δ*algK*(MCS-5:*algK-his*).

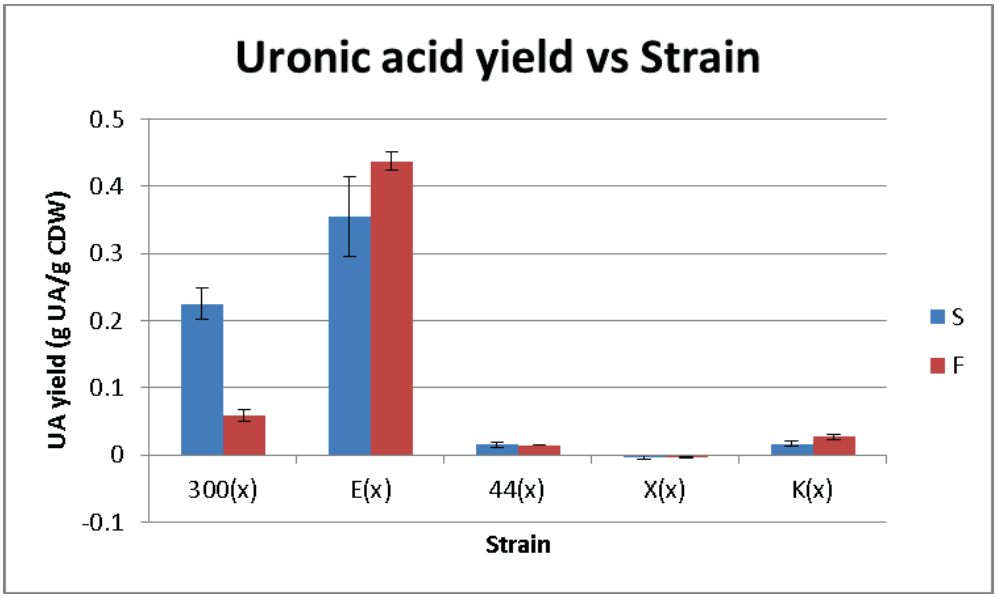

Figure 23. Determination of uronic acid concentration in unfiltered and filtered culture supernatants of PDO300 and its Δ*algE*, *44*, *X* and *K* mutants. Strains were inoculated from overnight cultures into 50 ml PI media with relevant antibiotics to an initial O.D $_{600nm}$ of 0.05 and cultivated at 37°C for 24 h. Uronic acid content of unfiltered culture supernatants (S) and filtered supernatants (F) (at 10 kDa cut off) was determined as described in Sections 2.9.2 and 2.9.3. Background uronic acid of growth media was determined and subtracted from values. Values of uronic acid yield were standardised to cellular dry weight. Displayed values are averages and error bars represent standard deviations from three independent experiments. 300(x) = *P. aeruginosa* PDO300, E(x) = PDO300Δ*algE*, 44(x) = PDO300Δ*alg44*, X(x) = PDO300Δ*algX*, and K = PDO300Δ*algK*. All strains have empty miniCTX-2 plasmids integrated and excised from *attB* site of chromosome (x).

3.2.3 Sub-cellular localisation of AlgK protein

Immunoblot analysis (Section 2.10.8) of envelope fractions from various strains (Section 2.10.4) was carried out to determine the localisation of AlgK and to assess whether the deletion of the *algK* gene abolished the presence of the AlgK protein. This was done for strains grown on solid and in liquid media. AlgK was present in the envelope fractions of the wild type strains PDO300(CTX) and PDO300(MCS-5) but absent in that of the *algK* mutants Δ*algK*(CTX) and Δ*algK*(MCS-5) (Figure 24). Introducing the *algK* ORF or *algK-his in trans* into the Δ*algK* mutant restored the presence of AlgK (Figure 24). However, *in cis* complementation only recovered AlgK during biofilm but not during planktonic growth (Figure 24).

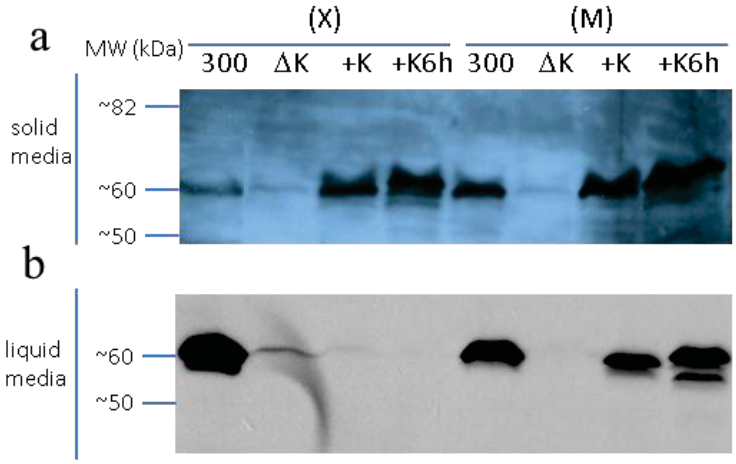

Figure 24. Localisation of AlgK in the envelope fractions of various strains. Strains were grown on solid PIA media for 72 h (a) or in liquid PI media for 14 h (b). Envelope fractions were prepared and subjected to immunoblot analysis with primary rabbit anti-AlgK antibodies followed by secondary goat anti-rabbit antibodies conjugated to horse radish peroxidase (Section 2.10.8). Bound secondary antibodies were visualised as described in Section 2.10.8. (x) = strains harbouring miniCTX-2 derived plasmids, (m) = strains harbouring pBBR1MCS-5 derived plasmids, 300 = *P. aeruginosa* PDO300 containing empty miniCTX-2 (x) or pBBR1MCS-5 (m) vectors, ΔK = PDO300Δ*algK* containing empty miniCTX-2 (x) or pBBR1MCS-5 (m) vectors, +K = Δ*algK* mutant complemented with *algK* ORF *in cis* (x) or *in trans* (m), and +K-his = Δ*algK* mutant complemented with *algK-his in cis* (x) or *in trans* (m).

3.2.4 The role of AlgK in mutual stability of subunits

Immunoblot analyses were performed to investigate the role of AlgK in the stability of other subunits of the alginate biosynthesis machinery (e.g. AlgE, Alg44 and AlgX), and to assess whether the absence of various subunits would destabilise AlgK and other components of this complex. The strains used in this study were PDO300 and its Δ*alg44*, Δ*algE*, Δ*algX* and Δ*algK* mutants as well as their respective *in cis* complemented strains (Gutsche et al. 2006, Remminghorst & Rehm 2006a, Hay et al. 2010). The effect of growth mode on protein stability was also evaluated by conducting these experiments on strains cultivated in liquid (Figure 25) and on solid (Figure 26) media.

Regardless of growth mode, all proteins were detectable in PDO300(CTX) (Lane 1 of Figures 25 & 26). During planktonic growth, the absence of Alg44 in the Δ*alg44* mutant resulted in the loss of AlgX and AlgK while subsequent *in cis* complementation of the Δ*alg44* mutant restored Alg44, AlgX and AlgK to detectable levels (Figure 25, lanes 2 & 3). When cultivated in liquid media, the loss of AlgE in the Δ*algE* mutant also abolished the presence of AlgX and AlgK, and *in cis* complementation recovered the presence of AlgE, AlgX and AlgK (Figure 25, lanes 4 & 5). The absence of AlgX in the Δ*algX* mutant during planktonic growth was correlated with the absence of Alg44, AlgE and AlgK, and introducing the *algX* gene *in cis* restored Alg44, AlgE, AlgX and AlgK (Figure 25, lanes 6 & 7). The loss of AlgK in the Δ*algK* mutant was accompanied by the absence of Alg44 and AlgX and a reduction of AlgE during growth in liquid media (Figure 25, lane 8). Interestingly, complementation failed to restore Alg44, AlgX and AlgK to detectable levels during this growth mode; however, the presence of AlgE was increased relative to the Δ*algK* mutant (Figure 25, lane 9).

When cultivated on solid media, the loss of Alg44 in the Δ*alg44* mutant was associated with the disappearance of AlgX and AlgK while subsequent *in cis* complementation of the Δ*alg44* mutant returned Alg44, AlgX and AlgK to detectable levels (Figure 26, lanes 2 & 3). Similarly, the absence of AlgE in the Δ*algE* mutant was accompanied by the loss of AlgX and a reduction of AlgK while complementation restored the presence AlgE, AlgX and AlgK (Figure 26, lanes 4 & 5). Interestingly, the loss of AlgX in the

Δ*algX* mutant during biofilm growth did not appear to have affected the stability of other subunits (Figure 26, lane 6). Introducing the *algX* gene *in cis* recovered the presence of AlgX (Figure 26, lane 7). During growth on solid media, the absence of AlgK in the Δ*algK* mutant was associated with the reduced presence of AlgX while introducing the *algK* gene *in cis* was sufficient to restore both proteins to detectable levels (Figure 26, lanes 8 & 9).

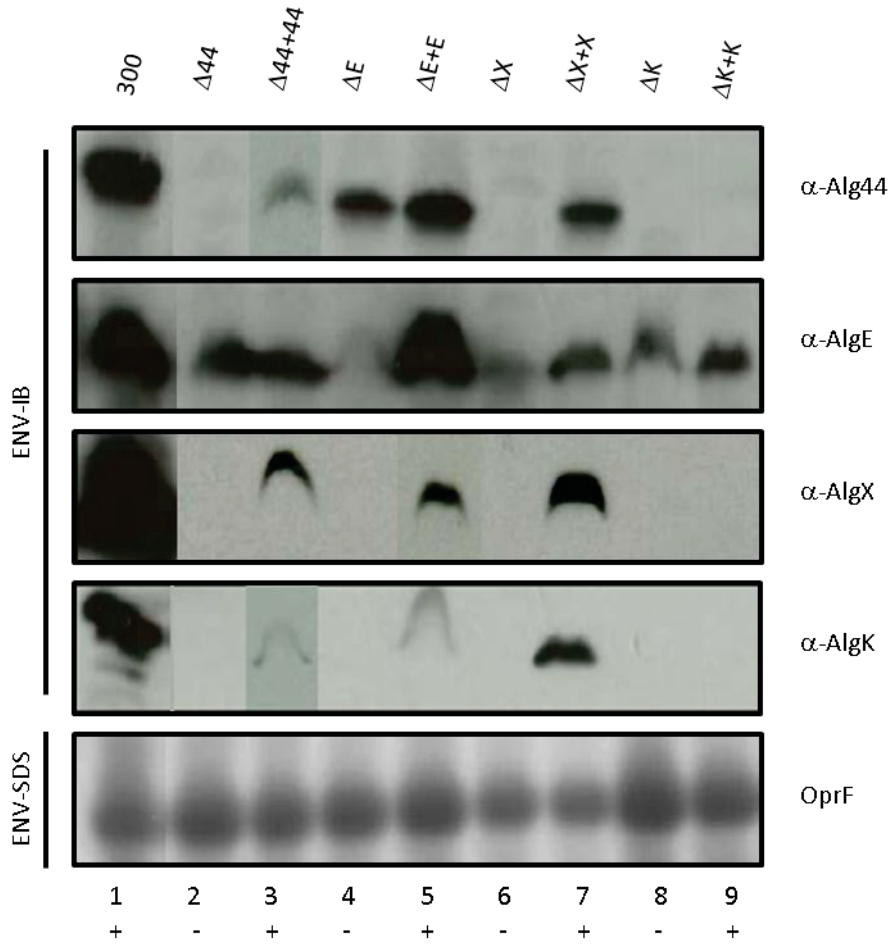

Figure 25. Effect of the absence or presence of proposed subunits of the alginate biosynthesis machinery on the stability of other subunits in the multi-protein complex during planktonic growth. PDO300 (lane 1) and its isogenic marker free mutants, Δ*alg44*, Δ*algE*, Δ*algX* and Δ*algK* (lanes 2, 4, 6 and 8) as well as their respective *in cis* complemented strains (lanes 3, 5, 7 and 9) were grown for 14 h in planktonic mode. Envelope fractions were prepared and subjected to immunoblot analysis using antibodies as indicated to detect the presence and absence of various subunits of the alginate biosynthesis machinery. Only relevant parts of the blots are shown. ENV-IB = Immunoblot of envelope fractions, and ENV-SDS = SDS-PAGE of envelope fractions. Constitutively expressed outer membrane protein OprF was used as a loading control. (+) and (-) for each strain indicate that alginate was detectable and undetectable, respectively. Immunoblot analysis was performed by Dr. Zahid Rehman.

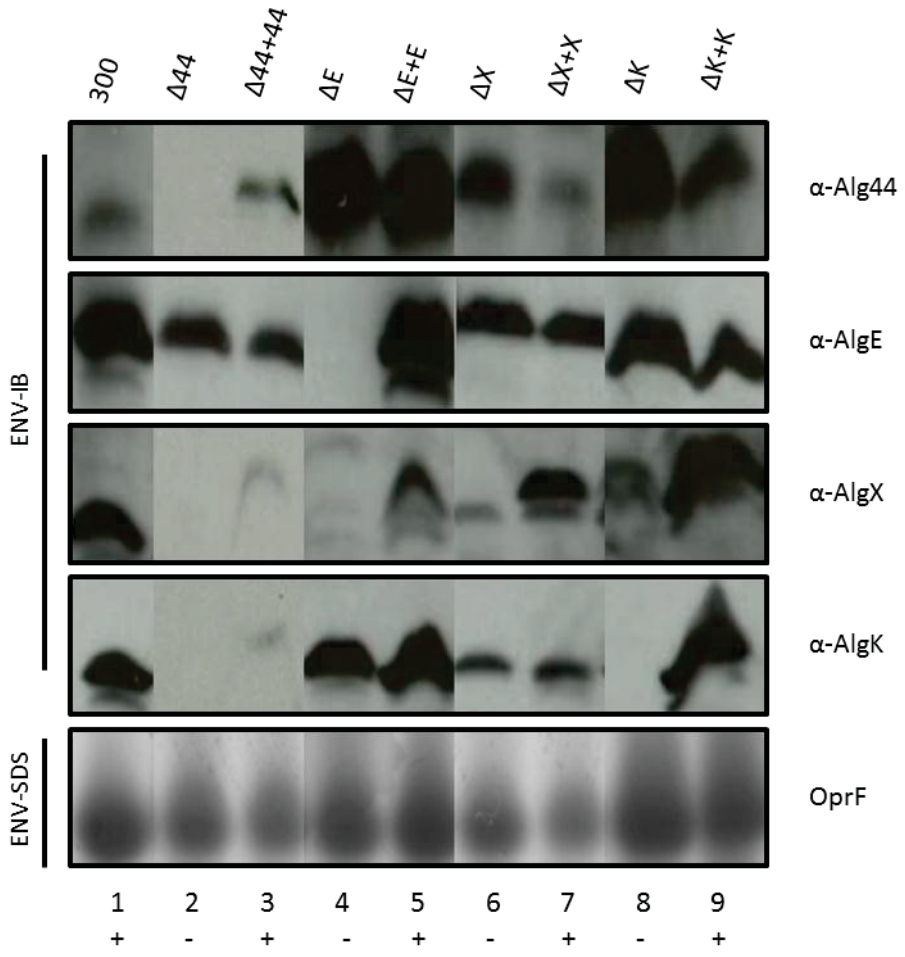

Figure 26. Effect of the absence or presence of proposed subunits of the alginate biosynthesis machinery on the stability of other subunits in the multi-protein complex during growth on solid media. PDO300 (lane 1) and its isogenic marker free mutants, Δ*alg44*, Δ*algE*, Δ*algX* and Δ*algK* (lanes 2, 4, 6 and 8) as well as their respective *in cis* complemented strains (lanes 3, 5, 7 and 9) were grown on solid PIA media for 72 h. Envelope fractions were prepared and subjected to immunoblot analysis using antibodies as indicated to detect the presence and absence of various subunits of the alginate biosynthesis machinery. Only relevant parts of the blots are shown. ENV-IB = Immunoblot of envelope fractions, and ENV-SDS = SDS-PAGE of envelope fractions. Constitutively expressed outer membrane protein OprF was used as a loading control. (+) and (-) for each strain indicate that alginate was detectable and undetectable, respectively. Immunoblot analysis was performed by Dr. Zahid Rehman.

3.2.5 Identifying PPI partners of AlgK via pull down experiments

Our mutual stability results (Section 3.2.4) indicated that AlgK may be required for the stability of Alg44, AlgX and AlgE (Figures 25 & 26, lanes 8 & 9). Furthermore, they demonstrated that the stability of AlgK was reduced in the absence of Alg44, AlgX and AlgE in their respective mutants (Figures 25 & 26), suggesting that these proteins may interact with AlgK. To investigate this further, pull down experiments followed by immunoblot analyses were conducted. These experiments utilised the *in trans* complemented strains Δ*algK*(MCS-5:*algK*) and Δ*algK*(MCS-5:*algK-his*) because they produced AlgK during both planktonic and biofilm growth modes (Figure 24). These strains were preferred over the *in cis* complemented strains for the reason that the latter strains did not produce AlgK during planktonic growth (Figure 24).

The strains Δ*algK*(MCS-5:*algK*) and Δ*algK*(MCS-5:*algK-his*) were cultivated in liquid PI and on solid PIA media (2.2.3) containing gentamycin (2.3). Cells were harvested, disrupted, and the envelope fractions were prepared, solubilised and subject to nickel affinity purification (Sections 2.10.1 to 2.10.5). The eluted samples were then run on SDS-PAGE (Section 2.10.7), transferred to a nitrocellulose membrane and probed with relevant antibodies (Section 2.10.8). After a series of optimization experiments, suitable conditions were found (Figure 27). In these experiments, each stage of purification was probed with anti-His antibodies to detect the AlgK-His protein. As expected, AlgK-His was only purified from Δ*algK*(MCS-5:*algK-his*) but not from the negative control Δ*algK*(MCS-5:*algK*) (Figure 27). Specificity of the anti-His antibody was also confirmed by probing a His-tagged protein molecular weight ladder (Figure 27, lane L).

Under the optimised conditions, a crosslinking step was introduced prior to cell disruption (Section 2.10.2). This was done based on the assumption that the crosslinkers could penetrate into the periplasm and 'lock' interaction partners together. Initial experiments incorporating crosslinkers were performed on cells grown on solid media because this mimicked the biofilm growth phase when alginate production was at its highest level, theoretically maximising the number of intact alginate biosynthesis machinery. Crosslinked samples were subject to nickel affinity purification under denaturing conditions to increase purification efficiency while inhibiting proteolysis (Sections 2.10.4 to 2.10.5). However, crosslinking was ineffective, regardless of growth

mode. Consequently, this step was abandoned and denaturing conditions were replaced with non-denaturing conditions because in the absence of effective crosslinking, denaturing conditions would abolish all protein interactions.

AlgK-His was present in the eluted fraction of Δ*algK*(MCS-5:*algK-his*) but not in that of Δ*algK*(MCS-5:*algK*) (Figure 28a). The eluted fractions were probed with anti-AlgX, anti-AlgE and anti-Alg44 antibodies (Figure 28b-d). Positive (+) and negative (Δ) controls were included to show the specificity of the relevant antibody. In the case of the anti-AlgX antibody, the corresponding positive and negative controls were the envelope fractions of PDO300Δ*algX*(MCS-5:*algX*) and PDO300Δ*algX*(MCS-5); as expected, AlgX was present in the positive and absent in the negative control (Figure 28b). AlgX was also found in the eluted fraction of Δ*algK*(MCS-5:*algK-his*) but not in that of Δ*algK*(MCS-5:*algK*), providing strong evidence that AlgK interacted with AlgX (Figure 28b). However, AlgE and Alg44 did not co-purify with AlgK-His, suggesting that AlgK did not interact with AlgE or Alg44 *in vivo* (Figure 28c-d). The positive and negative controls (+E and ΔE, and +44 and Δ44) indicated that the anti-AlgE and anti-Alg44 antibodies were specific (Figure 28c-d). An additional band in lane +K-his in Figure 28c raised speculation that AlgE could interact with AlgK; however, this experiment would need to be repeated, possibly under further optimised conditions, to support this claim.

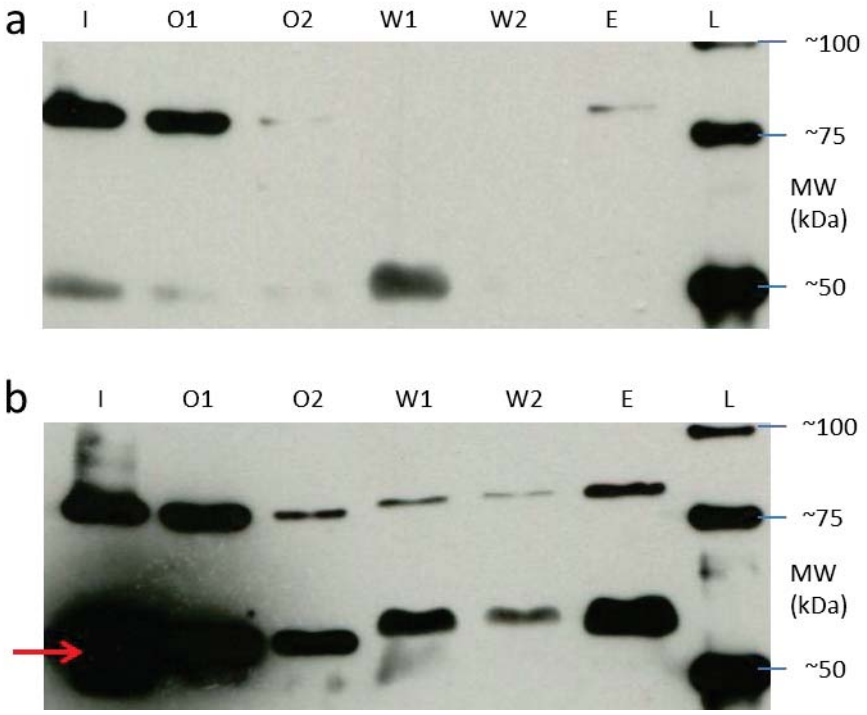

Figure 27. Pull down optimization. The strains (a) Δ*algK*(MCS-5:*algK*) (negative control) and (b) Δ*algK*(MCS-5:*algK-his*) were grown on solid media and envelope fractions were prepared and solubilised under non-denaturing conditions in Buffer B1 and then subject to nickel affinity purification using His-Spin Protein Miniprep^{TM} (Zymo Research, USA) with non-denaturing Buffer System B. A sample from each stage of purification was probed with Anti-His antibody to detect the C-terminally hexahistidine tagged AlgK protein (AlgK-His). I = input (solubilised envelope fraction); O1 and O2 = output (flow through after 1st and 2nd incubation with affinity gel, respectively); W1 and W2 = flow through after washing with wash buffers containing 25 and 50 mM imidazole, respectively; E = eluted sample with elution buffer containing 500 mM imidazole; L = 6xHis Protein MW Ladder (Qiagen, USA), and red arrow = expected MW of AlgK-His protein. Pull down experiments using planktonic cells and substituting non-denaturing (Buffer System B) for denaturing conditions (Buffer System A) yielded similar results (data not shown).

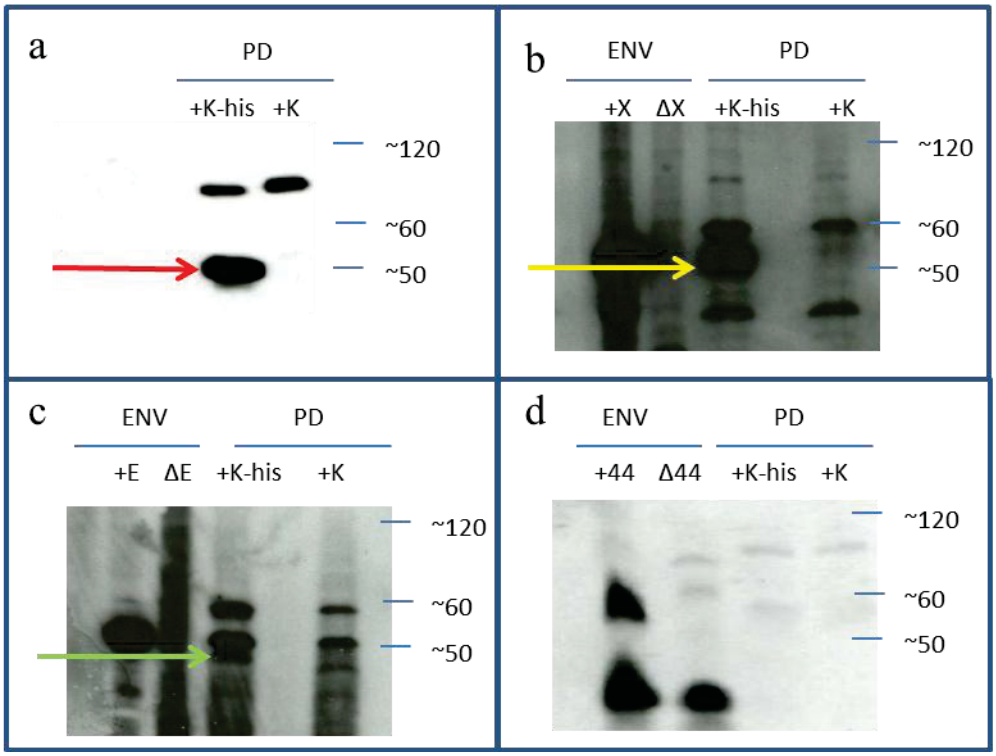

Figure 28. Immunoblot analysis for detection of various proteins in elution fractions of pull down experiments. Strains Δ*algK*(MCS-5:*algK-his*) and Δ*algK*(MCS-5:*algK*) were grown on solid media for 72 h. Envelope fractions were solubilised under non-denaturing conditions with Buffer B1 and then subject to nickel affinity purification using His-Spin Protein Miniprep™ (Zymo Research, USA) and non-denaturing Buffer System B. Eluted samples were probed by (a) anti-His, (b) anti-AlgX, (c) anti-AlgE and (d) anti-Alg44 antibodies. +K-his and +K = eluted fractions from PDO300Δ*algK*(MCS-5:*algK-his*) and PDO300Δ*algK*(MCS-5:*algK*), respectively; +X, +E, +44 = envelope fractions of the respective mutants Δ*algX*, Δ*algE* and Δ*alg44* complemented *in trans* with the affected gene on pBBR1MCS-5; ΔX, ΔE, Δ44 = envelope fractions of the respective mutants Δ*algX*, Δ*algE* and Δ*alg44* which contain the empty pBBR1MCS-5 vector; red arrow indicates AlgK-His protein; yellow arrow indicates AlgX protein; green arrow indicates suspected AlgE protein; ENV = envelope fractions and PD = pull down elution fractions. Protein molecular weights are shown in kDa.

CHAPTER FOUR

DISCUSSION

Alginate biosynthesis by *P. aeruginosa* is thought to be facilitated by a multi-protein complex spanning the envelope fraction. The alginate precursor, GDP-mannuronic acid, is synthesised in the cytoplasm by AlgA, AlgC and AlgD (Roychoudhury et al. 1989, Zielinski et al. 1991, May et al. 1994). GDP-mannuronic acid is polymerised by the inner membrane proteins Alg8 and Alg44, forming a poly-mannuronate chain which is translocated by a periplasmic scaffold consisting of AlgL, AlgG, AlgX and AlgK to the outer membrane porin, AlgE, for secretion (Jain & Ohman 1998, Jain et al. 2003, Robles-Price et al. 2004, Jain & Ohman 2005, Remminghorst & Rehm 2006a, Remminghorst & Rehm 2006b, Oglesby et al. 2008, Hay et al. 2010b). In the periplasm, polymannuronate can be modified through o-acetylation by AlgI, AlgJ and AlgF, epimerisation by AlgG, and degradation by AlgL (Franklin & Ohman 2002, Douthit et al. 2005, Bakkevig et al. 2005). According to this model, when components involved in polymerisation are missing, alginate is not produced (Remminghorst & Rehm 2006a, Remminghorst & Rehm 2006b, Oglesby et al. 2008). However, if components facilitating translocation or secretion are not present, alginate leaks into the periplasm where it is degraded by AlgL, releasing free uronic acid oligomers (Jain & Ohman 1998, Jain et al. 2003, Robles-Price et al. 2004, Hay et al. 2010b).

The aim of this study was to elucidate the role of AlgK in alginate biosynthesis. AlgK is a periplasmic protein anchored to the inner leaflet of the outer membrane by a lipid moiety (Aarons et al. 1997, Jain & Ohman 1998). It contains multiple TPR-like repeat motifs, a feature of proteins involved in assembly of multi-protein complexes (Keiski et al. 2010). This suggests that AlgK could be critical for assemblage of functional alginate biosynthesis machinery.

4.1 Molecular cloning

To investigate the role of AlgK in alginate biosynthesis, a well-established strategy was used to generate a defined isogenic marker free Δ*algK* deletion mutant in a constitutive alginate over-producer, *P. aeruginosa* PDO300 (Hoang et al. 1998). This strategy involved three steps. The first step was to construct a suicide vector, pEX100TΔ*algK*ΩGm^R, designed to disrupt the *algK* gene. This plasmid has two key features: (1) a counter selectable *sacB* gene encoding an enzyme which converts sucrose into poly-fructose, a toxic metabolite, and (2) a cassette designed to disrupt the *algK* gene, *algKN*-FRT-*aacC1*-FRT-*algKC*, which consists of a gentamycin resistance marker (*aacC1*) flanked by FRT recognition sequences and an N and a C terminal region of the *algK* ORF. The plasmid was introduced into PDO300 in the second step under gentamycin and sucrose selection for a double cross over event. Gentamycin selects for the *aacC1* gene of the cassette while sucrose selects against the *sacB* gene of the plasmid backbone. Under these conditions, cells would undergo a double cross over event for survival by replacing the middle of the *algK* ORF in the chromosome with the gentamycin resistance marker while discarding the rest of the plasmid harbouring the *sacB* gene. In the final step the marker was removed by introducing the flip recombinase encoding plasmid, pFLP2 (Hoang et al. 1998). Successful homologous recombination (generating PDO300Δ*algK*ΩGm^R) and subsequent removal of the gentamycin resistance marker (generating PDO300Δ*algK*) were confirmed by PCR, and the gain and loss of gentamycin resistance (Figure 14).

The strategy used to generate PDO300Δ*algK* has several distinct advantages. First, the mutant generated was defined because only a single gene is affected (Hoang et al. 1998). Second, the parent strain, PDO300, is genetically defined (Stover et al. 2000). PDO300 is derived from the sequence determined PAO1 strain through allelic exchange; it carries a mutant *mucA22* allele encoding a truncated MucA protein which cannot sequester AlgU, leading to constitutive alginate over-production (Mathee et al. 1997). Third, the mutant was isogenic ensuring that the expression of downstream genes was not compromised. Fourth, the mutant was marker free; hence, it did not need to be constantly exposed to antibiotic selection which would elevate alginate production as a protective response (Bagge et al. 2004, Wood et al. 2006). Finally, the absence of an additional promoter ensured that expression of downstream genes was not artificially

elevated. Although this technique left an FRT scar within the disrupted gene, a number of researchers have successfully used it for generating isogenic marker free Δ*algE,* Δ*algX,* Δ*alg44* and Δ*alg8* mutants without any problems (Gutsche et al. 2006, Remminghorst & Rehm 2006a, Remminghorst & Rehm 2006b, Hay et al. 2010b).

To complement the Δ*algK* mutant, the *algK* ORF and *algK-his* were introduced *in trans* and *in cis*. *In trans* complementation was performed because it was the most commonly used method. However, the gene was controlled by a constitutively expressing *lacZ* promoter on a medium copy number plasmid, pBBR1MCS-5, which could raise copy number issues (Kovach et al. 1995). Thus, *in cis* complementation was also employed. Although more complex than *in trans* complementation, this strategy allows a single copy of *algK* (or *algK-his*) to be integrated in the genome under the control of its native promoter (P*alg*) in the absence of antibiotic resistance selection. The complemented strains and control strains were successfully generated and subsequently confirmed by PCR and restriction analysis (Figures 14 & 18).

4.2 Requirement of AlgK for alginate biosynthesis

The Δ*algK* mutant generated in this study was non-mucoid and did not produce alginate or the AlgK protein (Figures 20, 21 & 24). Introducing the *algK* ORF or *algK-his* into the Δ*algK* mutant was sufficient to restore mucoidity, alginate production and the AlgK protein, indicating that the Δ*algK* mutant was isogenic (Figures 20, 21 & 24). These results also demonstrated that AlgK was essential for alginate biosynthesis, a finding consistent with previous studies (Aarons et al. 1997, Jain & Ohman 1998).

To determine at which stage AlgK participated in alginate biosynthesis, we measured the yield and size distribution of uronic acids released by the Δ*algK* mutant, and its parent and complemented strains when grown in planktonic mode (Figure 22). We quantified the uronic acid content of (i) the culture supernatant corresponding to the sum of high molecular weight alginate and alginate degradation products and (ii) the culture supernatant after filtration (10 kDa cut off) equating to all the alginate degradation products, herein referred to as free uronic acid oligomers. PDO300 produced high molecular weight alginate, of which approximately a quarter was

degraded. We showed that the Δ*algK* mutant yielded background levels of free uronic acid oligomers (Figure 22). This suggested that AlgK played a role in polymerisation. Intriguingly, expressing the *algK* ORF or *algK-his in trans* or *in cis* in the Δ*algK* mutant failed to restore alginate production to PDO300 levels. However, a substantial proportion of the uronic acid perceived in the culture supernatants of the complemented strains was high molecular weight, suggesting that complementation had restored function to some extent (Figure 29). In this study, PDO300 and the complemented strains produced significantly less uronic acid during planktonic than biofilm growth (Figures 21 & 22), indicating that alginate production is disfavourable during planktonic growth, a finding in concordance with earlier research (Hassett 1996, Pena et al. 2000).

The inability of the Δ*algK* mutant and its complemented strains to produce high quantities of uronic acids during planktonic growth – regardless of size - is contrary to what has been previously reported (Jain & Ohman 1998). These authors generated an Δ*algK* mutant which produced large quantities of free uronic acid oligomers and a complemented strain that yielded alginate at levels comparable to wild type. We suggest that this difference is attributed to the dissimilarity in methods of mutant generation and complementation. In the present study, a region of the *algK* gene was deleted without leaving an antibiotic resistance marker while in Jain & Ohman (1998), an Δ*algK* mutant was engineered by replacing the whole *algK* gene with a gentamycin resistance cassette containing a promoter which would artificially drive the transcription of downstream genes. This extra promoter could elevate the expression of *algA* which encodes an enzyme that participates in alginate precursor (uronic acid) synthesis.

In our study, the Δ*algK* mutant was complemented by introducing only the *algK* ORF (or *algK-his*), whereas in their investigation, two genes, *alg44* and *algK*, were re-introduced for complementation (Jain & Ohman 1998). Remminghorst & Rehm (2006a) have previously shown that extra copies of the *alg44* gene can significantly elevate alginate production which could explain why their complemented strains synthesised much more alginate than ours. Furthermore, the difference in parent strains (PDO300 vs. FRD1) and growth media could have also contributed to the discrepancies observed between our studies. However, how and to what extent these factors contributed to this disparity is unknown.

This peculiar observation could also be explained by a disruption of stoichiometry. The balance hypothesis proposes that the stoichiometry of subunits within a multi-protein complex must be kept consistent in order to maintain biological function (Papp et al. 2003). Thus, any imbalance caused by the absence, reduction or elevated presence of a subunit could destabilise and disrupt the function of the whole complex. This has been widely observed in a number of multi-protein complexes (Alani et al. 1997, Ouspenski et al. 1999, Jose et al. 2012). In the present study, the copy number of AlgK in the complemented strains appeared to be less than in PDO300 during planktonic growth (Figure 24). Assuming that PDO300 produced each subunit of the alginate biosynthesis machinery at its optimal level, then any deviation from this optimum - in this case due to the deletion of *algK* and inefficient expression of the AlgK protein after complementation - could destabilise the complex and terminate alginate production.

In this study, the alginate yield and size distribution of several other mutants derived from PDO300, Δ*alg44*, Δ*algE* and Δ*algX*, were also examined in parallel to the Δ*algK* mutant and PDO300 (Figure 23). PDO300(CTX) produced reasonable amounts of high molecular weight alginate. By comparison, none of the Δ*algE,* Δ*alg44*, Δ*algX* and Δ*algK* mutants produced high molecular weight alginate. Instead, the Δ*algE* mutant yielded high quantities of free uronic acid oligomers, indicating that polymerisation was not affected in the absence of AlgE, a finding consistent with previous reports (Figure 23, Hay et al. 2010b). In contrast, the Δ*algK,* Δ*alg44* and Δ*algX* mutants released background levels of free uronic acid oligomers, indicating that AlgK, Alg44 and AlgX were necessary for polymerisation (Figure 23). This is in concordance with earlier studies which have shown that Alg44 is essential for polymerisation (Remminghorst & Rehm 2006a, Oglesby et al. 2008). However, literature has been more divided regarding the role of AlgX. While studies by Robles-Price et al. (2004) proposed that AlgX exclusively participated in alginate translocation and secretion, a more recent investigation by Gutsche et al. (2006) suggested that AlgX was required for efficient polymerisation. In their study Gutsche et al. (2006) generated an Δ*algX* mutant which produced ~98% less uronic acid than its parent strain, FRD1, during planktonic growth. Similarly, in the present study our Δ*algX* mutant produced approximately 20 folds less uronic acid than its parent strain, PDO300 (Figure 22).

4.3 Mutual stability

Mutual stability experiments were performed to assess whether the absence of AlgK affected the stability of other subunits of the alginate biosynthesis machinery and to determine whether the absence of individual components would affect the stability of AlgK and each other. Mutual stability is based on the assumption that if two proteins interact, then the absence of one protein would reduce or abolish the presence of the other. The same mutants used for the free uronic acid assay (PDO300Δ*alg44*, Δ*algX*, Δ*algE* and Δ*algK*) were employed in the mutual stability experiments. Previous studies have shown that expressing a gene *in trans* on a medium copy number plasmid could have adverse effects on protein stability due to copy number issues (Medina et al. 2002). Therefore, in the present study, we used *in cis* complemented strains for mutual stability analysis.

Our results indicate that components of the alginate biosynthesis machinery (AlgE, Alg44, AlgX and AlgK) appeared to be more stable and/or produced in greater quantities during biofilm growth compared to planktonic growth (Figures 25 & 26). This observation was in concordance with the elevated alginate yield observed during biofilm growth (Figures 21 & 22), suggesting that more alginate biosynthesis machineries are active during this growth mode. While all proteins were detectable in the envelope fraction of the alginate over-producer, PDO300(CTX), upon the deletion of a gene, alginate production was abolished along with the presence of the respective gene product and at least one other protein which could be an interaction partner (Figures 22, 25 & 26). Subsequent *in cis* complementation with the affected gene was sufficient to restore alginate production and the presence of all proteins (Alg44, AlgE, AlgX and AlgK) in the envelope fraction (Figures 21, 25 & 26). The exception to this trend was the *in cis* complemented Δ*algK* mutant which only produced AlgE but not Alg44, AlgX or AlgK at detectable levels during planktonic growth. Further studies may be required to determine why this was the case.

Regardless of growth mode, the absence of either the inner membrane protein Alg44 or the outer membrane protein AlgE in their respective mutants (Δ*alg44* and Δ*algE*) was sufficient to destabilise the periplasmic proteins AlgX and AlgK (Figures 24 & 25, lanes 2 & 4). This is consistent with a recent study by Rehman & Rehm (2013) who

demonstrated that AlgE was required for the stability of AlgK, AlgX and Alg44. In the present study, the absence of AlgX or AlgK in their respective mutants (Δ*algX* and Δ*algK*) was sufficient to destabilise both proteins along with Alg44 and AlgE during planktonic growth (Figure 25, lanes 6 & 8). However, during biofilm growth, the stability of Alg44 and AlgE was not affected in the Δ*algX* and Δ*algK* mutants (Figure 26, lanes 6 & 8), suggesting that Alg44 and AlgE were more stable and/or produced more strongly during biofilm growth. Our results also indicated that AlgK and AlgX were strongly interdependent for stability; in nearly all cases, the absence of one of these proteins was linked to the absence or reduced presence of the other (Figures 25 & 26, rows "α-AlgK" & "α-AlgX"). This suggested that AlgK could interact with AlgX *in vivo*.

Overall, our mutual stability experiments demonstrate that the absence of individual components of the alginate biosynthesis machinery brought instability to the whole complex. On the basis of the mutual stability data, we proposed two putative three-way protein-protein interactions that may occur *in vivo*: AlgE-AlgK-AlgX and Alg44-AlgX-AlgK. Conceivably, these groups would be intimately associated with the outer and inner membranes through AlgE and Alg44 and overlap in the periplasm through AlgK and AlgX. This model is in concordance with current understanding that alginate is synthesised by a multi-protein complex spanning the entire envelope fraction.

4.4 Protein-protein interaction

To test the two putative three-way protein-protein interactions proposed from the mutual stability data, we performed pull down and immunoblot analyses using the *in trans* complemented strains PDO300Δ*algK*(MCS-5:*algK*) and Δ*algK*(MCS-5:*algK-his*). Given that alginate yield and apparent stability of the alginate biosynthesis machinery were greater during biofilm growth, we used cells grown on solid media for initial pull down experiments. After optimisation suitable conditions were found (Figure 27). Under these conditions the AlgK-His protein was reproducibly detectable in the eluted fraction of Δ*algK*(MCS-5:*algK-his*) but not in that of Δ*algK*(MCS-5:*algK*) (Figures 27 & 28a).

Under the optimised conditions, we introduced a crosslinking step. However, our attempts using crosslinkers on cells harvested from solid media were unsuccessful. This was thought to be due to the extracellular material, such as alginate, preventing the penetration of the crosslinkers into the periplasm; hence, we repeated the experiment with planktonic cells, which produced less alginate. This also proved difficult, suggesting that the crosslinking chemistry was incompatible for AlgK and its interaction partners. These crosslinkers, DSG and DSP, form amide bonds between primary amines of lysine residues. Although AlgK has 17 lysine residues available for crosslinking, the spacer arm length, 7.7 Å for DSP and 12 Å for DSG, may have been too short to allow efficient crosslinking between distant lysine residues of AlgK and its interaction partners. As a result, the crosslinking step was abandoned.

AlgK was found to interact with AlgX (Figure 28b), a finding consistent with previous studies (Gutsche et al. 2006, Hay et al. 2012). Recently, AlgX was also shown to interact with MucD, a negative regulator of alginate biosynthesis (Hay et al. 2012). Given its association with MucD through AlgX, AlgK could also have regulatory roles in alginate biosynthesis. Future studies could explore this possibility.

Our mutual stability results using the Δ*algK* mutant were consistent with previous studies which have indicated that AlgK is required for the proper localisation of AlgE, suggesting an interaction between these proteins (Keiski et al. 2010). Furthermore, homologues of AlgK and AlgE involved in PGA biosynthesis by *Yersinia pestis,* HmsH and HmsF, respectively, have been shown to interact (Abu Khweek et al. 2010). To examine whether AlgK interacted with AlgE, we probed the eluted fractions from pull down experiments with anti-AlgE antibodies (Figure 28c). However, under our experimental conditions, no interaction between AlgK and AlgE was demonstrated.

AlgK has also been suggested to interact with Alg44 (Keiski et al. 2010). Similar to AlgK, Alg44 is required for the stability of AlgE (Oglesby et al. 2008). Alg44 has a periplasmic C-terminal domain similar to membrane fusion proteins (MFP) of multi-drug efflux pumps, suggesting that it could interact with periplasmic and/or outer membrane components of the alginate biosynthesis machinery (Oglesby et al. 2008, Merighi et al. 2007, Remminghorst & Rehm 2006a). However, Keiski et al. (2010) have suggested that the MFP domain of Alg44 may not be long enough to extend across the

periplasm and interact directly with AlgE. Hence, Keiski et al (2010) have proposed that Alg44 could indirectly interact with AlgE through a periplasmic protein such as AlgK or AlgX. Our mutual stability data using Δ*algK* and Δ*algX* mutants were consistent with this hypothesis (Figures 25 & 26). To investigate whether AlgK interacted with Alg44, the eluted fractions from pull down assays were probed with anti-Alg44 antibodies (Figure 28d). However, no interaction between AlgK and Alg44 was identified.

4.5 Future directions

This study indicates that AlgK is important for the stability of Alg44, AlgX and AlgE. The interaction between AlgK and AlgX observed in this study should be further verified with another method such as the yeast two-hybrid system. Future optimisation of pull down experiments may be required to reveal any interaction between AlgK and AlgE or Alg44. For instance, we could use other detergents or try enzymatic cell lysis to conserve the integrity of membrane fractions; we used French Press for lysis which could be harsh on membranes. Site directed mutagenesis can also be employed to identify key residues facilitating these interactions.

Previous studies have demonstrated that the absence or over-expression of various subunits of the alginate biosynthesis machinery can alter the extent of epimerisation and acetylation (Robles-Price et al. 2004, Douthit et al. 2005, Remminghorst & Rehm 2006a). The effect of the absence of AlgK on alginate structure, degree of epimerisation and acetylation should also be explored. These experiments can be performed in conjunction with mutual stability experiments using newly generated *algG, algI, algJ* and *algF* mutants to assess whether AlgK is required for the stability and function of the proteins facilitating the epimerisation and acetylation.

Previously, it was demonstrated that increased *algD* promoter activity was associated with the deletion or over production of various subunits of the alginate biosynthesis machinery (Hay et al. 2012). Future studies could examine the effect of deleting/over expressing *algK* on the *algD* promoter activity which could reveal whether AlgK has an additional regulatory role.

As we become increasingly familiar about the molecular mechanisms behind the regulation and biosynthesis of alginate, we can start to rationally design inhibitors to eliminate *P. aeruginosa* infections by blocking alginate production. It will also allow us to develop custom designed, commercially synthesised bacterial alginate long awaited for by medical and biotechnological industries.

4.6 Summary

In this study, an isogenic marker free Δ*algK* deletion mutant was generated. This mutant was successfully complemented by independently introducing the *algK* gene and *algK-his in trans* and *in cis*. We show that AlgK is required for alginate biosynthesis and appears to be essential for polymerisation. We also reveal that AlgK is important for the stability of key components of the alginate biosynthesis machinery Alg44, AlgX and AlgE, supporting the hypothesis that AlgK is critical for assembly of functional alginate biosynthesis machinery. Furthermore, we demonstrate through pull down experiments that AlgK interacts with AlgX. However, no interaction between AlgK and Alg44 or AlgE is identified.

APPENDIX I

Uronic acid assay

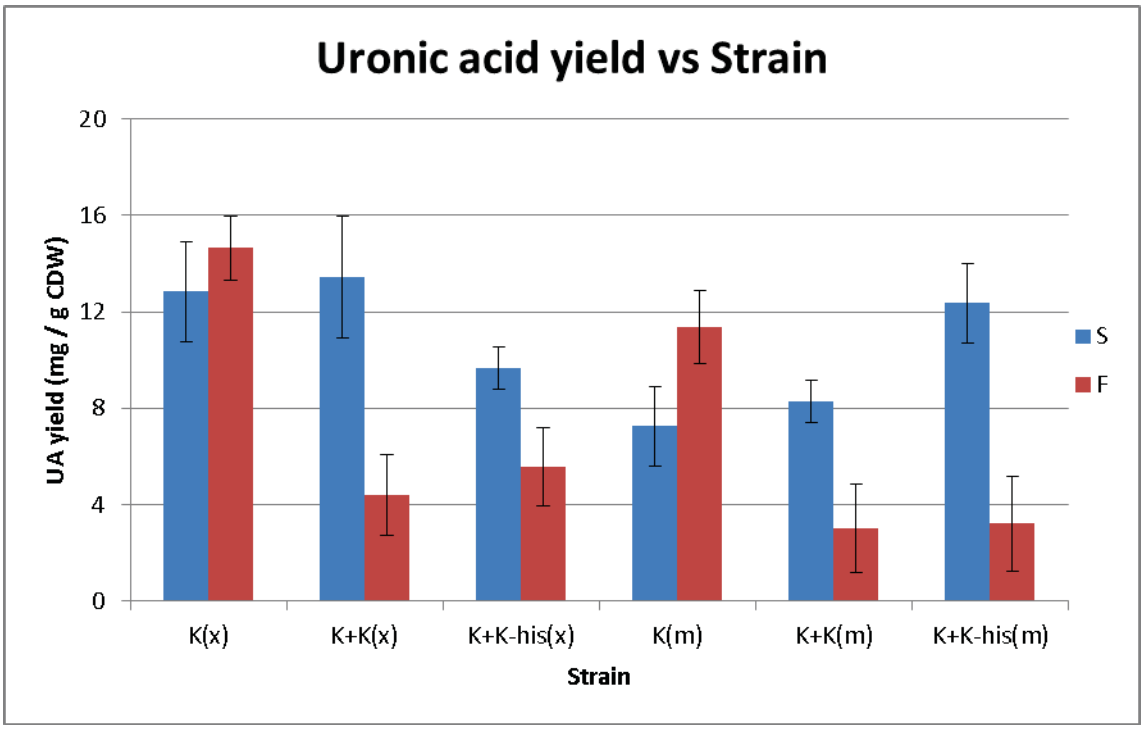

Figure 29. Determination of uronic acid concentration in unfiltered and filtered culture supernatants of *algK* mutant and complemented strains. Strains were inoculated from overnight cultures into 50 ml PI media with relevant antibiotics to an initial O.D $_{600nm}$ of 0.05 and cultivated at 37°C for 24 h. Uronic acid content of unfiltered culture supernatants (S) and filtered supernatants (F) (at 10 kDa cut off) was determined as described in Sections 2.9.2 and 2.9.3. Background uronic acid of growth media was determined and subtracted from values. Values of uronic acid yield were standardised to cellular dry weight. Displayed values are means and error bars represent standard deviations from three independent experiments. K = PDO300Δ*algK*. K+K and K+K-his = PDO300Δ*algK* complemented with the *algK* ORF and *algK* gene C-terminally fused to a hexahistidine tag, respectively. (x) and (m) = strains complemented *in cis* and *in trans*, respectively.

APPENDIX II

PUBLICATION

The current study has contributed to the following publications:

Rehman, Z. U., **Wang, Y**., Moradali, M. F., Hay, I. D., Rehm, B. H., (2013) Insight into assembly of the alginate biosynthesis machinery in *Pseudomonas aeruginosa*. Appl Environ Microbiol 79: 3264-3272.

Hay, I. D., Rehman, Z. U., Moradali, M. F., **Wang, Y**., Rehm, B. H., (2013) Microbial alginate production, modification and its applications. *Microbial Biotechnology*, MICROBIO-2013-042. Submitted.

REFERENCES

Aarons, S. J., Sutherland, I. W., Charkabarty, A. M., & Gallagher, M. P. (1997) A novel gene, *algK*, from the alginate biosynthesis cluster of *Pseudomonas aeruginosa*. Microbiol 143: 641-652.

Abu Khweek, A., Fetherston, J. D., & Perry, R. (2010) Analysis of HmsH and its role in plague biofilm formation. Microbiol 156: 1424-1438.

Agren, M. S. (1999) Zinc in wound repair. Arch Dermatol 135: 1273-1274.

Alani, E., Sokolsky, T., Studamire, B., Miret, J. J., & Lahue, R. S. (1997) Genetic and biochemical analysis of Msh2p-Msh6p: role of ATP hydrolysis and Msh2p-Msh6p subunit interactions in mismatch base pair recognition. Mol Cell Biol 17: 2436-2447.

Alban, S., Schauerte, A., & Franz, G. (2002) Anticoagulant sulfated polysaccharides: part I. Synthesis and structure-activity relationships of new pullulan sulphates. Carbohyd Polym 47: 267-276.

Albrecht, M. T., & Schiller, N. L. (2005) Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol 187: 3869-3872.

Alemayehu, D., Casey, P. G., McAuliffe, O., Guinane, C. M., Martin, J. G., Shanahan, F., et al. (2012) Bacteriophages phi MR299-2 and phi NH-4 can eliminate *Pseudomonas aeruginosa* in the murine lung and on cystic fibrosis lung airway cells. mBio 3: 10.1128/mBio.00029-12.

Amikam, D., & Galperin, M. Y. (2006) PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinfomatics 22: 3-6.

Bagge, N., Schuster, M., Hentzer, M., Ciofu, O., Givskov, M., Greenberg, E. P., et al. (2004) *Pseudomonas aeruginosa* biofilms exposed to imipenem exhibit changes in global gene expression and beta-lactamase and alginate production Antimicrob Agents Ch 48: 1175-1187.

Bakkevig, K., Sletta, H., Gimmstad, M., Aune, R., Ertesvag, H., Degnes, K., et al. (2005) Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment. J Bacteriol 187: 8375-8384.

Baynham, P. J., Ramsey, D. M., Gvozdvev, B. V., Cornnier, E. M., & Wozniak, D. J. (2006) The *Pseudomonas aeruginosa* ribbon-helix-helix DNA-bind protein

AlgZ (AmrZ) controls twitching motility and biogenesis of type IV pilli. J Bacteriol 188: 132-140.

Bjerkan, T. M., Lillehov, B. E., Strand, W. I., Skjak-Braek, G., Valla, S., & Ertesvag, H. (2004) Construction and analyses of hybrid *Azotobacter vinelandii* mannuronan C-5 epimerases with new epimerization pattern characteristics. Biochem J 381: 813-821.

Blumenkrantz, N., & Asboe-Hansen, G. (1973) New method for quantitative determination of uronic acids. Anal Biochem 54: 484–489.

Boles, B. R., & Singh, P. K. (2008) Endogenous oxidative stress produces diversity and adaptability in biofilm communities. P Natl Acad Sci USA 105: 12503–12508.

Boles, B. R., Thoendel, M., & Singh, P. K. (2005) Genetic variation in biofilms and the insurance effects of diversity. Microbiol 151: 2816–2818

Boucher, J. C., Schurr, M. J., Yu, H., Rowen, D. W., & Deretic, V. (1997) *Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity. Microbiol 143: 3473–3480.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Bragonzi, A., Paroni, M., Nonis, A., Cramer, N., Montanari, S., Rejman, J., et al. (2009) *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am J Resp Crit Care 180: 138–145.

Cezairliyan, B., & Sauer, R. T. (2009) Control of *Pseudomonas aeruginosa* AlgW protease cleavage of MucA by peptide signals and MucB. Mol Microbiol 72: 368-379.

Choi, K. H., Kumar, A., & Schweizer, H. P. (2006) A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. J Micobiol Meth 64: 391-397.

Chu, L., May, T. B., Chakrabarty, A. M., & Misra, T. K. (1991) Nucleotide sequence and expression of the *algE* gene involved in alginate biosynthesis by *Pseudomonas aeruginosa*. Gene 107: 1-10.

Coban, A. Y., Ciftci, A., Onuk, E. E., Erturan, Z., Cayci, Y. T., & Durupinar, B. (2009) Investigation of biofilm formation and relationship with genotype and antibiotic

susceptibility of *Pseudomonas aeruginosa* strains isolated from patients with cystic fibrosis. Mikrobiyol Bul 43: 563-573.

Coleman, R. J., Lawrie, G., Lambert, L. K., Whittaker, M., Jack, K. S., & Grondahi, L. (2011) Phosphorylation of alginate: synthesis, characterization, and evaluation of *in vitro* mineralization capacity. Biomacromolecules 12: 889-897.

Cystic Fibrosis Mutation Database. (2011) *Cystic Fibrosis Mutation Database*. Retrieved February 18, 2013, from http://www.genet.sickkids.on.ca/ Home.html.

"Cystic fibrosis signs & symptoms." *NIH Heart, Lung and Blood Institute*. Retrieved February 12, 2013, from http://www.nhlbi.nih.gov/health//dci/Diseases/cf/cf_signs.html.

Damron, F. H., & Goldberg, J. B. (2012) Proteolytic regulation of alginate overproduction in *Pseudomonas aeruginosa.* Mol Microbiol 84: 595–607.

Damron, F. H., Qiu, D., & Yu, H. W. (2009) The *Pseudomonas aeruginosa* sensor kinase KinB negatively controls alginate production through AlgW-dependent MucA proteolysis. J Bacteriol 191: 2285-2295.

Douthit, S. A., Dlakic, M., Ohman, D. E., & Franklin, M. J. (2005) Epimerase active domain of *Pseudomonas aeruginosa* AlgG, a protein that contains a right-handed beta-helix-fold. J Bacteriol 187: 4573-4583.

Draget, K., Smidsrod, O., & Skjak-Braek, G. (2005) Alginates from Algae. Biopolymers Online.

Firoved, A. M., Boucher J. C., & Deretic, V. (2002) Global genomic analysis of AlgU (sigma(E))-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. J Bacteriol 184: 1057–1064.

Franklin, M. J., Douthit, S. A., & McClure, M. A. (2004) Evidence that the *algI*/*algJ* gene cassette, required for acetylation of *Pseudomonas aeruginosa* alginate, evolved by lateral gene transfer. J Bacteriol 186: 4759-4773.

Franklin, M. J., & Ohman, D. E. (1996) Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. J Bacteriol 178: 2186-2195.

Franklin, M. J., & Ohman, D. E. (2002) Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O-acetylation of alginate in *Pseudomonas aeruginosa*. J Bacteriol 184: 3000-3007.

Friedrich, B., Hogrefe, C., & Schlegel, H. G. (1981) Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J Bacteriol 147:198-205.

Gacesa, P. (1998) Bacterial alginate biosynthesis - recent progress and future prospects. Microbiol-UK 144: 1133-1143.

Gimmestad, M., Sletta, H., Ertesvag, H., Bakkevig, K., Jain, S., Suh, S. J., et al. (2003) The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J Bacteriol 185: 3515-3523.

Goh, C. H., Heng, P. W., & Chan, L. W. (2012) Alginates as a useful natural polymer for microencapsulation and therapeutic applications. Carbohyd Polym 88: 1-12.

Gutsche, J., Remminghorst, U., & Rehm, B. H. (2006) Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie 88: 245-251.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166: 557-580.

Harmsen, M., Yang, L. A., Pamp, S. J., & Tolk-Nielsen, T. (2010) An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. Fems Immunol Med Mic 59: 253-268.

Hartmann, M., Dentini, M., Draget, K. I., & Skjak-Braek, G. (2006) Enzymatic modification of alginates with the mannuronan C-5 epimerase AlgE4 enhances their solubility at low pH. Carbohyd Polym 63: 257-262.

Hassett, D. J. (1996) Anaerobic production of alginate by *Pseudomonas aeruginosa*: Alginate restricts diffusion of oxygen. J Bacteriol 178: 7322-7325.

Hay, I. D., Rehman, Z. U., Ghafoor, A., & Rehm, B. H. (2010a) Bacterial biosynthesis of alginates. J Chem Technol Biot 85: 752-759.

Hay, I. D., Rehman, Z. U., & Rehm, B. H., (2010b) Membrane topology of outer membrane protein AlgE, which is required for alginate production in *Pseudomonas aeruginosa*. Appl Environ Microb 76: 1806-1812.

Hay, I. D., Remminghorst, U., & Rehm, B. H., (2009) MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Appl Environ Microb 75: 1110-1120.

Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., & Schweizer, H. P. (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of

chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene 28: 77-86.

Hoang, T. T., Kutchma, A. J., Becher, A., & Schweizer, H. P. (2000) Integration-proficient plasmids for *Pseudomonas aeruginosa:* site-specific integration and use for engineering of reporter and expression strains. Plasmid 43: 59-72.

Itoh, Y., Rice, J. D., Goller, C., Pannuri, A., Taylor, J., Meisner J., et al. (2008) Roles of *pgaABCD* genes in synthesis, modification, and export of the *Escherichia coli* biofilm adhesin poly-beta-1,6-N-acetyl-D-glucosamine. J Bacteriol 190: 3670-3680.

Jain, S., Franklin, M. J., Ertesvag, H., Valla, S., & Ohman, D. E. (2003) The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol Microbiol 47: 1123-1133.

Jain, S., & Ohman, D. E. (1998) Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J Bacteriol 180: 634-641.

Jain, S., & Ohman, D. E. (2005) Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infect Immun 73: 6429-6436.

Jao, W., Chen, H. C., Lin, C. H., & Yang, M. C. (2009) The controlled release behavior and pH- and thermo-sensitivity alginate/poly(vinyl alcohol) blended hydrogels. Polym Advan Technol 20: 680-688.

Jerga, A., Raychaudhuri, A., & Tipton, P. A. (2006a) *Pseudomonas aeruginosa* C5-mannuronan epimerase: steady-state kinetics and characterization of the product. Biochemistry 45: 552-560.

Jerga, A., Stanley, M. D., & Tipton, P. A. (2006b) Chemical mechanism and specificity of the C5-mannuronan epimerase reaction. Biochemistry 45: 9138-9144.

Jinek, M., Rehwinkel, J., Lazarus, B. D., Izaurralde, E., Hanover, J. A., & Conti, E. (2004) The superhelical TPR-repeat domain of O-linked GlcNAc transferase exhibits structural similarities to importin alpha. Nat Struct Mol Biol 11: 1001–1007.

Jose, D., Weitzel, S. E., Jing, D., & von Hippel, P. H. (2012) Assembly and subunit stoichiometry of the functional helicase-primase (primosome) complex of bacteriophage T4. P Natl Acad Sci USA 109: 13596:13601.

Kato, J., Misra, T. K., & Chakrabarty, A. M. (1990) AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. P Natl Acad Sci USA 87: 2887–2891.

Keiski, C. L., Harwich, M., Howell, P. L., Jain, S., Neculai, A. M., Yip, P., et al. (2010) AlgK Is a TPR-containing protein and the periplasmic component of a novel exopolysaccharide secretin. Structure 18: 265-273.

Kennedy, M. B. (1995) Origin of PDZ(DHR,GLGF) domains. Trends Biochem Sci 20: 464-468.

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., et al. (1995) Four new derivatives of the broadhost-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166: 175–176.

Kumar, V. (2007) *Robbins basic pathology*. Philadelphia: Saunders/Elsevier.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lim, G. J., Zare, S., Dyke, M. V., & Atala, A. (2010) Cell microencapsulation. Adv Exp Med Biol 670: 126-136.

Lizewski, S. E., Schurr, J. R., Jackson, D. W., Frisk, A., Carterson, A. J., & Schurr, M. J. (2004) Identification of *algR*-regulated genes in *Pseudomonas aeruginosa* by use of microarray analysis. J Bacteriol 186: 5672-5684.

Lobedanz, S., Bokma, E., Symmons, M. F., Koronakis, E., Hughes, C., & Koronakis, V. (2007) A periplasmic coiled-coil interface underlying TolC recruitment and the assembly of bacterial drug efflux pumps. P Natl Acad Sci USA 104: 4612-4617.

Luthy, L., Grutter, M. G., & Mittl, P. R. (2004) The crystal structure of *Helicobacter* cysteine rich protein C at 2.0 A resolution: similar peptide-binding sites in TPR and SEL1-like repeat proteins. J Mol Biol 340: 829–841.

Ma, S., Selvaraj, U., Ohman, D. E., Quarless, R., Hassett, D. J., & Wozniak, D. J. (1998) Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. J Bacteriol 180: 956-968.

Maharaj, R., May, T. B., Wang, S. K., & Chakrabarty, A. M. (1993) Sequence of the Alg8 and Alg44 genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene 136: 267-269.

Mak, Y. M. & Ho, K. K. (1992) An improved method for the isolation of chromosomal DNA from various bacteria and cyanobacteria. Nucleic Acid Res 20: 4101-4102.

Mann, E. E., & Wozniak, D. J. (2012) *Pseudomonas* biofilm matrix composition and niche biology. FEMS Microbiol Rev 36: 893-916.

Mathee, K., Ciofu, O., Sternberg, C., Lindum, P. W., Campbell, J. I., Jensen, P., et al. (1999) Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiol 145: 1349-1357.

Mathee, K., McPherson, C. J., & Ohman, D. E. (1997) Posttranslational control of the *algT* (algU)-encoded sigma(22) for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). J Bacteriol 179: 3711-3720.

Mathee, K., Narasimhan, G., Valdes, C., Qiu, X., Matewish, J. M., Koehrsen, M., et al. (2008) Dynamics of *Pseudomonas aeruginosa* genome evolution. P Natl Acad Sci USA 105: 3100–3105.

May, T. B., Shinabarger, D., Boyd, A., & Chakrabarty, A. M. (1994) Incorporation of isotope from specifically labelled glucose into alginates of *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. J Bacteriol 158: 1161-1162.

Medina, M. G., Carbonell, X., & Villaverde, A. (2002) Connection between gene dosage and protein stability revealed by a high-yield production of recombinant proteins in an *E. coli* LexA1(Ind(-)) background. Biotechnol Bioeng 78: 722-730.

Mena, A., Smith, E. E., Burns, J. L., Speert, D.P, Moskowitz, S. M., Perez, J. L., et al. (2008) Genetic adaptation of *Pseudomonas aeruginosa* to the airways of Cystic Fibrosis patients is catalysed by hypermutation. J Bacteriol 190: 7910-7917.

Merighi, M., Lee, V. T., Hyodo, M., Hayakawa, Y., & Lory, S. (2007) The second messenger bis-(3'-5')-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. Mol Microbiol 65: 876-895.

Microsoft Office - Microsoft Word, Outlook & Excel - Office.com. *Office - Office.com*. Retrieved February 11, 2013, from http://office.microsoft.com/en-NZ/?CTT=97

Moreau-Marquis, S., Stanton, B. A., & O'Toole, G. A. (2008) *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. Pulm Pharmacol Ther 21: 595-599.

Mrsny, R. J., Daugherty, A. L., Short, S. M., Widmer, R., Siegel, M. W., & Keller, G. A. (1996) Distribution of DNA and alginate in purulent cystic fibrosis sputum: implications to pulmonary targeting strategies. J Drug Target 4: 233-243.

Muhammadi, M., & Ahmed, N. (2007) Genetics of bacterial alginate: alginate genes distribution, organization and biosynthesis in bacteria. Curr Genomics 8: 191-202.

Nivens, D. E., Ohman, D. E., Williams, J., and Franklin, M. J (2001) Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J. Bacteriol. 183: 1047-1057.

Oglesby, L. L., Jain, S., & Ohman, D. E. (2008) Membrane topology and roles of *Pseudomonas aeruginosa* Alg8 and Alg44 in alginate polymerization. Microbiol 154: 1605-1615.

O'Sullivan, B. P., & Freedman, S. D. (2009) Cystic Fibrosis. Lancet 373: 1891-1904.

Ouspenski, I. I., Elledge, S. J., & Brinkley, B. R. (1999) New yeast genes important for chromosome integrity and segregation identified by dosage effects on genome stability. Nucleic Acids Res 27: 3001-3008.

Paletta, J. L., & Ohman, D. E. (2012) Evidence for two promoters internal to the alginate biosynthesis operon in *Pseudomonas aeruginosa*. Curr Microbiol 65: 770-775.

Papp, B., Pal, C., & Hurst, L. D. (2003) Dosage sensitivity and the evolution of gene families in yeast. Nature 424: 194-197.

Pedersen, S. S., Kharazmi, A., Espersen, F., & Hoiby, N. (1990) *Pseudomonas aeruginosa* alginate in Cystic Fibrosis sputum and the inflammatory response. Infect Immun 58: 3363–3368.

Pena, C., Trujillo-Roldan, M. A., & Galindo, E. (2000) Influence of dissolved oxygen tension and agitation speed on alginate production and its molecular weight in cultures of *Azotobacter vinelandii*. Enzyme Microb Tech 27: 390-398.

Pier, G. B., Coleman, F., Grout, M., Franklin, M., & Ohman, D. E. (2001) Role of alginate O-acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. Infect Immun 69: 1895-1901.

Pineda, M., Gregory, B. D., Szczypinski, B., Baxter, K. R., Hochschild, A., Miller, E. S., et al. (2004) A family of anti-sigma(70) proteins in T4-type phages and bacteria that are similar to AsiA, a transcription inhibitor and co-activator of bacteriophage T4. J Mol Biol 344: 1183-1197.

Qin, Y. M. (2008) Alginate fibres: an overview of the production processes and applications in wound management. Polym Int 57: 171-180.

Qiu, D., Damron, F. H., Mima, T., Schweizer, H., & Yu, H. D. (2008a) PBAD-based shuttle vectors for functional analysis of toxic and highly-regulated genes in *Pseudomonas* and *Burkholderia* spp. and other bacteria. Appl Environ Microbiol 74: 7422–7426.

Qiu, D., Eisinger, V. M., Head, N. E., Pier, G. B., & Yu, H. D. (2008b) ClpXP proteases positively regulate alginate overexpression and mucoid conversion in *Pseudomonas aeruginosa*. Microbiol 154: 2119–2130.

Qiu, D., Eisinger, V. M., Rowen, D. W., & Yu, H. W. (2007) Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. P Natl Acad Sci USA 104: 8107-8112.

Ramsey, D. M., Baynham, P. J., & Wozniak, D. J. (2005) Binding of *Pseudomonas aeruginosa* AlgZ to sites upstream of the *algZ* promoter leads to repression of transcription. J Bacteriol 187: 4430-4443.

Rehm, B. H. (2009) Alginates: biology and applications. Berlin: Springer-Verlag.

Rehm, B. H. (2010) Bacterial polymers: biosynthesis, modifications and applications. Nat Rev Microbiol 8: 578-592.

Rehm, B. H., Boheim, G., Tommassen, J., & Winkler, U. K. (1994) Overexpression of *algE* in *Escherichia coli*: subcellular localisation, purification, and ion channel properties. J Bacteriol 176: 5639-5647.

Rehm, B. H., & Valla, S. (1997) Bacterial alginates: biosynthesis and applications. App Microbiol Biot 48: 281-288.

Rehman, Z. U., & Rehm, B. H. (2013) The dual roles of Pseudomonas aeruginosa AlgE in secretion of the virulence factor, alginate, and formation of the secretion complex. Appl Environ Microbiol. .03960-12; published ahead of print 18 January 2013.

Rehman, Z. U., Wang, Y., Moradali, M. F., Hay, I. D., Rehm, B. H., (2013) Insight into assembly of the alginate biosynthesis machinery in *Pseudomonas aeruginosa*. Appl Environ Microbiol: AEM00460-13R2. Accepted.

Remminghorst, U., Hay, I. D., & Rehm, B. H. (2009) Molecular characterization of Alg8, a putative glycosyltransferase, involved in alginate polymerisation. J Biotechnol 140: 176-183.

Remminghorst, U., & Rehm, B. H. (2006a) Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. FEBS Let 580: 3883-3888.

Remminghorst, U., & Rehm, B. H. (2006b) *In vitro* alginate polymerization and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. Appl Environ Microbio 72: 298-305.

Robles-Price, A., Wong, T. Y., Sletta, H., Valla, S. & Schiller, N. L. (2004) AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol 186: 7369-7377.

Romling, U., Gomelsky, M., & Galperin, N. Y. (2005) C-di-GMP: the dawning of a novel bacterial signalling system. Mol Microbiol 57: 629-639.

Rowe, S. M., Miller, S., & Sorscher, E. J. (2005) Mechanisms of disease: cystic fibrosis. New Engl J Med 352: 1992-2001.

Rowley, G., Spector, M., Kormanec, J., & Roberts, M. (2006) Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. Nat Rev Microbiol 4: 383–394.

Roychoudhury, S., May, T. B., Gill, J. F., Singh, S. K., & Feingold, D. S. (1989) Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. A key enzyme in the biosynthesis of alginate by *Pseudomonas aeruginosa*. J Biol Chem 264: 9380-9385.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schlegel, H. G., Kaltwasser, H., & Gottschalk, G. (1961) [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies] article in German. *Arch Mikrobiol* 38: 209-222.

Selden, C., & Hodgson, H. (2004) Cellular therapies for liver replacement. Transpl Immunol 12: 273-288.

Shinabarger, D., Berry, A., May, T. B., Rothmel, R., Fialho, A., & Chakrabarty, A. M. (1991) Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J Biol Chem 266: 2080-2088.

Simon, R., Priefer, U., & Puhler, A. (1983) A broad host range mobilization system for in vitro genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnol 1: 784–791.

Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., et al. (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. P Natl Acad Sci USA 103: 8487–8492.

Steward, A. J., Liu, Y., & Wagner, D. R. (2011) Engineering cell attachments to scaffolds in cartilage tissue engineering. JOM-US 63: 74-82.

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoquchi, S. D., Warrener, P., Hickey, M. J., et al. (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 31: 959-964.

Stutts, M. J., Canessa, C. M., Olsen, J. C., Hamrick, M., Cohn, J. A., Rossier, B. C., et al. (1995) CFTR as a cAMP-dependent regulator of sodium-channels. Science 269: 847-850.

Symmons, M. F., Bokma, E., Koronakis, E., Huges, C., & Koronakis, V. (2009) The assembled structure of a complete tripartite bacterial multidrug efflux pump. P Natl Acad Sci USA 106: 7173-7178.

Tatnell, P. J., Russell, N. J., & Gacesa, P. (1994) GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolic studies. Microbiol 140: 1745-1754.

Thomas, A., Harding, K.G., & Moore, K. (2000) Alginates from wound dressings activate human macrophages to secrete tumor necrosis factor-α. Biomaterials 21: 1797-1802.

Tonnesen, H. H., & Karlsen, J. (2002) Alginate in drug delivery systems. Drug Dev Ind Pharm 28: 621-630.

Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002) Extracellular DNA required for bacterial biofilm formation. Science 295: 1487.

Whitney, J. C., Hay, I. D., Li, C., Eckford, P. D., Robinson, H., Amaya, M. F., et al. (2011) Structural basis for alginate secretion across the bacterial outer membrane. P Natl Acad Sci USA 108: 13083-13088.

Wiggins, C. A., & Munro, S. (1998) Activity of the yeast MNN1 alpha-1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. P Natl Acad Sci 95: 7945-7950.

Williams, B. J., Dehnbostel. J., & Blackwell, T. S. (2010) *Pseudomonas aeruginosa*: Host defence in lung disease. Respirology 19: 1037-1056.

Wilkinson, L. J., White, R. J., & Chipman, J. K. (2011) Silver and nanoparticles of silver in wound dressings: a review of efficacy and safety. J Wound Care 20: 543-549.

Wong, T. Y., Preston, W. A., & Schiller, N. L. (2000) Alginate Lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. Annu Rev Microbiol 54: 289-240.

Wood, L. F., Leech, A. J., & Ohman, D. E. (2006) Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. Mol Microbiol 62: 412–426.

Wood, L. F., & Ohman, D., E. (2006) Independent regulation of MucD, an HtrA-like protease in *Pseudomonas aeruginosa*, and the role of its proteolytic motif in alginate gene regulation. J Bacteriol 88: 3134–3137.

Wood, L. F., & Ohman, D. E. (2009) Use of cell wall stress to characterize sigma(22) (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. Mol Microbiol 72: 183-201.

Wozniak, D. J., & Ohman, D. E. (1993) Involvement of the alginate *algU* gene and integration host factor in the regulation of *Pseudomonas aeruginosa algB* gene. J Bacteriol 175: 4145-4153.

Wozniak, D. J., Sprinkle, A. B., & Baynham, P. J. (2003) Control of *Pseudomonas aeruginosa algZ* expression by the alternative sigma factor AlgT. J Bacteriol 185: 7297-7300.

Yang, Z. H., Jin, X. L., Rao, X. C., & Hu, F. Q. (2011) A natural antisense transcript regulates *mucD* gene expression and biofilm biosynthesis in *Pseudomonas aeruginosa*. Microbiol 80: 768-774.

Yuan, A. H., Gregory, B. D., Sharp, J. S., McCleary, K. D., Dove, S. L., & Hochschild, A. (2008) Rsd family proteins make simultaneous interactions with regions 2 and 4 of the primary sigma factor. Mol Microbiol 70: 1136-1151.

Zielinski, N. A., Chakrabarty, A. M., & Berry, A. (1991) Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. J Biol Chem 266: 9754-9763.

Zimbro, M. J., Power, D. A., Miller, S. M., Wilson, G. E., & Johnson, J. A. (Eds.) (2009) *Difco & BBL Manual, Manual of Microbiological Culture Media, 2nd Ed*. Sparks, MD: BD Diagnostics – Diagnostic Systems.