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**Understanding aspects of alginate
biosynthesis and regulation by *Pseudomonas
aeruginosa***

A thesis presented in partial fulfilment of the

requirements of the degree of

Doctor of Philosophy

in

Microbiology

at Massey University, Palmerston North,

New Zealand



**MASSEY
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Abstract

Alginate is a medically and industrially important polymer produced by seaweeds and certain bacteria. The bacterium *Pseudomonas aeruginosa* over-produces alginate during cystic fibrosis lung infections, forming biofilms, making the infection difficult to treat. Bacteria make alginate using membrane spanning multi-protein complexes. Although alginate biosynthesis and regulation have been studied in detail, there are still major gaps in knowledge. In particular, the requirement of AlgL (a periplasmic alginate degrading enzyme) and role played by MucR (an inner membrane c-di-GMP modulator) are not well understood. Here I show that AlgL and MucR are not essential for alginate production during biofilm growth. My findings suggest that while catalytically active AlgL negatively affects alginate production, expressing catalytically inactive AlgL enhances alginate yields. Furthermore, preliminary data show AlgL is not required for the stability or functionality of the alginate biosynthesis complex, suggesting that it is a free periplasmic protein dispensable for alginate production. These findings support the prediction that the primary function of AlgL is to degrade misguided alginate from the periplasm. For MucR, I show for the first time that its sensor domain mediates nitrate-induced suppression of alginate biosynthesis. This appears to occur at multiple levels in a manner only partially dependent on c-di-GMP signaling. These results indicate that MucR is associated with the negative effect of nitrate (and possibly denitrification) on alginate production. On the basis of these results, I propose a combination of nitrate (or denitrification intermediates), exogenous lyases and antimicrobial agents could be used to eliminate established chronic biofilm infections. Furthermore, catalytically inactive AlgL and/or homologs of MucR with disabled sensor motifs could be harnessed in non-pathogenic bacteria for producing tailor-made alginates.

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Dedication

This thesis is dedicated to my late-grandfather Jiheng Wang, a former Professor of Plant Breeding, who passed away on the 2nd of November 2016, aged 95.

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List of Abbreviations

¹ H-NMR	Proton nuclear magnetic resonance
ANOVA	Analysis of variance
APS	Ammonium persulfate
BSA	Bovine serum albumin
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
CLSM	Confocal laser scanning microscopy
DGC	diguanylate cyclase
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphates
DSG	disuccinimidyl glutarate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MOPS	(3-(N-morpholino)propanesulfonic acid)
NIAC	nickel ion affinity chromatography
O.D.	Optical density
PCR	Polymerase Chain Reaction
PDE	phosphodiesterase
poly-M	polymannuronic acid
RE	Restriction endonuclease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
SEC-MALLS	Size Exclusion Chromatography-multi-Angle Laser Light Scattering
SLIM	Site-directed, Ligase-Independent Mutagenesis
TBE	Tris/Borate/EDTA
TBST	Tris-buffered-saline + Tween 20
TEMED	Tetramethylethylenediamine
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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1. Chapter One: Introduction

Alginate is a collective term for a family of polysaccharides produced by brown seaweeds and bacteria (Hay et al. 2010a). As a major cell wall constituent of brown seaweeds, alginate plays an important structural role in algal tissues and is harvested for many industrial and medical applications (Schmid et al. 2016). For instance, it is used in foods, cosmetics, pharmaceuticals and medical products as a stabilizer/thickener and encapsulation/drug delivery agent, and more recently for regenerative therapy (Section 1.1).

Alginate also plays an important role in bacteria. For instance, in *Azotobacter* spp., it is required for formation of desiccation resistant cysts (Campos et al. 1996). Recently, an important Gram-positive bacterium, *Sulfobacillus thermosulfidooxidans*, has been found to secrete alginate during bioleaching of chalcopyrite (Yu et al. 2017). It is also produced by various species of *Pseudomonas* such as *Pseudomonas syringae*, *Pseudomonas putida* and *Pseudomonas fluorescens*. In *P. syringae* alginate is produced to increase its epiphytic fitness and resistance to desiccation (Yu et al. 1999).

The opportunistic human pathogen, *P. aeruginosa*, over-produces alginate during chronic cystic fibrosis (CF) lung infections, growing as a biofilm (cells embedded in self-secreted polymeric matrix) to evade antibiotics and host immune responses, clogging patients' airways, causing chronic inflammation, tissue damage, pulmonary deterioration and death (Hoiby et al. 2010). Owing to its medical importance, alginate biosynthesis and regulation in *P. aeruginosa* has been studied in great detail. It is also used as a general model to help understand biofilm formation, antibiotic resistance, and to develop strategies to eliminate biofilms which are a major challenge in both medical and industrial settings (del Pozo and Patel 2007; Langsrud et al. 2003).

In this chapter, I start by providing a brief overview of the chemical structure, properties, commercial production and applications of alginate (Section 1.1), and the challenges posed by *P. aeruginosa* in clinical and industrial settings (Sections 1.2 and 1.3). As the foundation for my PhD studies, I review the mechanisms behind the biosynthesis (Section 1.4) and regulation (Section 1.5) of alginate production in bacteria. I then outline the foci of the thesis, AlgL, a periplasmic alginate lyase (Section 1.6), and MucR, an inner membrane regulatory protein (Section 1.7), and then present my aims and research questions in Section 1.8.

1.1 Structure and applications of alginate

1.1.1 Structure and properties

Alginate is an anionic polysaccharide consisting of variable ratios of β -D-mannuronate (M) and its C-5 epimer α -L-guluronate (G) linked by 1–4 glycosidic bonds. In contrast to seaweed alginate, bacterial alginate is O-acetylated, offering increased shear-thinning properties (Skjakbraek et al. 1989). The chemical structure of alginate is illustrated in Figure 1.1 on page 3. Alginate is biocompatible and biodegradable, and has excellent gel-forming, scaffold-forming and viscosifying properties (Schmid et al. 2016).

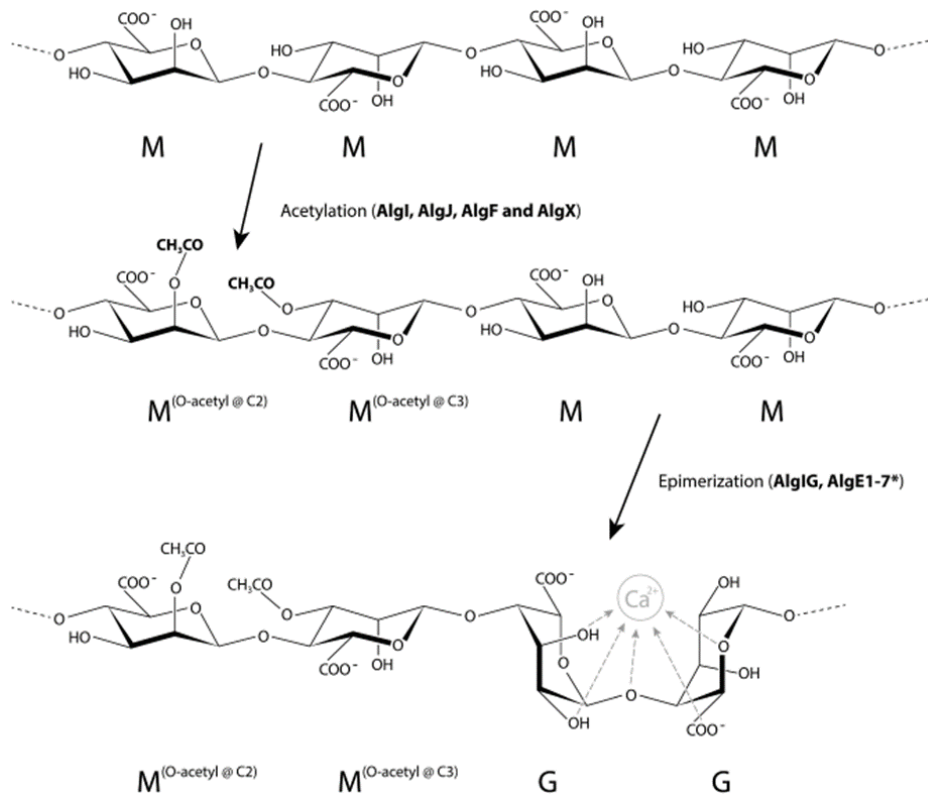


Figure 1.1 Chemical structure of alginate. It is composed of β -D-mannuronate (M) and its C-5 epimer α -L-guluronate (G) linked by 1–4 glycosidic bonds. They form MM, MG and GG blocks. The polymer can be O-acetylated (by AlgI, AlgJ, AlgF and AlgX) and M residues can be converted to G residues by epimerases (AlgG, AlgE1-7 of *Azotobacter* spp). Reuse permission obtained from John Wiley and Sons (Hay et al. 2013).

1.1.2 Extraction and applications

Each year 30,000 metric tons of alginate are harvested from brown seaweeds for numerous industrial and medical applications. Here I briefly describe the extraction process of alginate from seaweed, highlight some of the most recent applications for alginate, and emphasise the potential use of bacterial factories (or enzymes) to manufacture tailor-made alginate for high value medical applications.

The rationale behind extraction of alginate from seaweed is converting all alginate salts in the seaweed into the sodium salt, dissolving this in water, and removing the seaweed residue by filtration. The extraction process is summarised in Figure 1.2 on page 6. Firstly, the seaweed is chopped into pieces and mixed with sodium carbonate, obtaining a crude sodium alginate solution. After removal of insoluble debris, crude sodium alginate is enriched by precipitation with either calcium chloride or acid. Resulting calcium alginate/alginic acid fibres are further treated with sodium carbonate to obtain the enriched sodium alginate.

Alginate harvested from seaweed is used in many industrial and medical applications (Figure 1.3, page 9) (Norouzi et al. 2015; Ruvinov and Cohen 2016; Tavassoli-Kafrani et al. 2016; Venkatesan et al. 2015). Some of the most recent applications include alginate as an anticoagulation agent (Arlov and Skjak-Brak 2017), an encapsulation agent for delivery of probiotics (Jose Martin et al. 2015) and pesticides (Nuruzzaman et al. 2016), a safe and renewable material for food packaging (Tavassoli-Kafrani et al. 2016), and a recovery agent for critical, toxic and precious metals from the environment (Dodson et al. 2015).

In addition, alginate's mild gelation properties and hydrogel resemblance to extracellular matrix have made it an attractive vehicle for stem cell delivery (infused with bioactive molecules and regenerative factors) to regenerate damaged tissues

resulting from cardiac arrest (Ruvinov and Cohen 2016). Alginate is also used in wound dressings, skin substitutes and bioactive scaffolds – laced with growth factors, antibiotics and other medicines to encourage skin healing and regeneration (Kamoun et al. 2015; Norouzi et al. 2015). One recent exciting application for polysaccharides is the transplantation of the first synthetic trachea – developed using a novel composite scaffold containing alginate (Crowley et al. 2015).

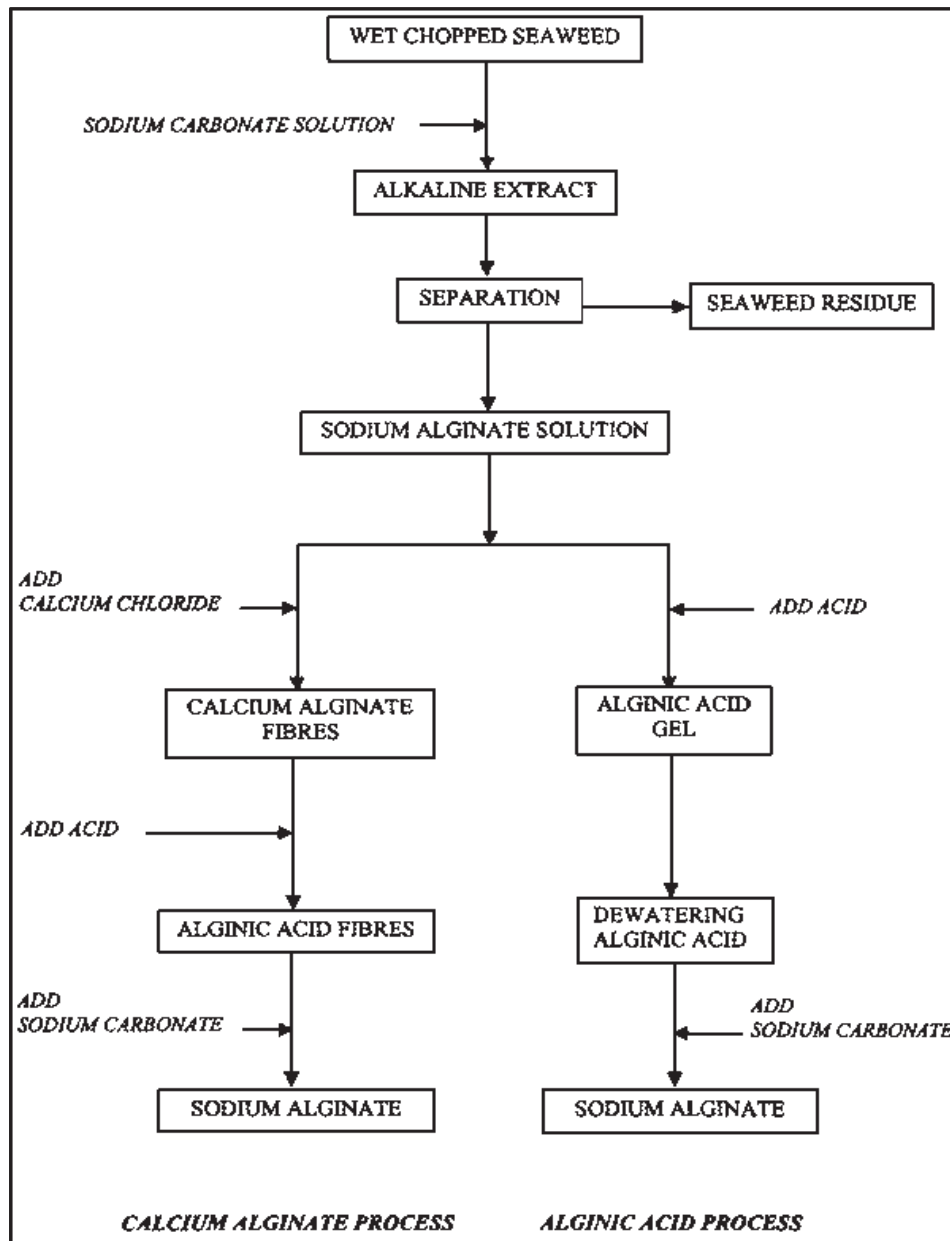


Figure 1.2. Isolation of alginate from seaweed. Seaweeds are harvested, chopped and converted to soluble sodium alginate by mixing with sodium carbonate. By filtration, residue seaweed is removed and discarded. The crude sodium alginate solution is further enriched by either the calcium alginate process or alginic acid process, where essentially the alginate is made insoluble by calcium ions or reduced pH. The alginate is then precipitated and dried into a paste and mixed with sodium carbonate again to obtain sodium alginate of greater purity.

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However, alginate from seaweed suffers from seasonal and environmental variation in molecular mass, composition and physicochemical properties (Rosell and Srivastava 1984; Saraswathi et al. 2003). For instance, different parts of the seaweed plant, different species, geographical location and season of harvesting can impact alginate properties (Haug et al. 1974). Such variability makes crude alginate isolated from seaweed cheap (\$US5 kg⁻¹). However, enriching pharmaceutical grade alginate of high purity and homogeneity in molecular weight (MW), mannuronic to guluronic acid (M/G) ratios and defined material properties from crude alginate is costly, with the end product reflecting this, costing up to \$US3,000 g⁻¹ (Rhein-Knudsen et al. 2015).

On the other hand, alginate-producing bacteria or bacterial alginate-modifying enzymes could be harnessed to produce alginate of defined properties, potentially alleviating this cost-burden. Using bacteria to produce commercial alginate is an attractive alternative because bacterial alginates are generally more homogeneous than that of seaweed; bacteria can be readily engineered and cultivated under controlled conditions to produce alginates of defined chemical structure and physicochemical properties. However, because of the potential pathogenic nature of *P. aeruginosa*, production of commercial bacterial alginates would most likely rely on *Azotobacter vinelandii* or non-pathogenic species of *Pseudomonas* genera.

While many breakthroughs have been made in understanding the mechanisms of bacterial alginate production and regulation, we are still far away from producing industrial quantities of pharmaceutical grade alginate of high purity, defined structure and physicochemical properties from bacteria. Nevertheless, pioneering studies have

developed strains and growth conditions offering reasonable control over alginate yield, molecular mass, composition and associated properties. For instance, *A. vinelandii* strains with increased transcription from alginate biosynthesis gene cluster have been engineered to increase alginate production levels. Moreover, shutting down of competing metabolic pathways and/or optimization of growth conditions (e.g. nutrients, oxygen availability) have also increased yields (e.g. up to 9.5 g Alg/L in 50 h) and improved control over alginate composition and properties (Bonartseva et al. 2017; Castillo et al. 2013; Diaz-Barrera et al. 2007; Diaz-Barrera et al. 2010; Flores et al. 2015; Gaytan et al. 2012; Pena et al. 2006). For example, a mutant of *P. fluorescens* lacking the epimerase function of AlgG has been generated that produces poly-M (Gimmestad et al. 2003). Furthermore, O-acetylation levels can also be controlled by using specific strains/mutants or altering the growth media and cultivation conditions, including aeration, pH and temperature (Diaz-Barrera et al. 2010; Gaytan et al. 2012; Pena et al. 2006).

Nature has provided a plethora of alginate modifying enzymes (reviewed in detail by (Ertesvag 2015)), including epimerases, O-acetyl-transferases and depolymerases that could be harnessed to modify seaweed alginates. For instance, *A. vinelandii* produces several epimerases, each introducing specific ratios and patterns of G residues (Gimmestad et al. 2003). These enzymes are used to modify alginate and can be used for immobilization of living tissue (Morch et al. 2007). Moreover, *P. syringae* has been used to O-acetylate seaweed alginate (Lee and Day 1995). Thus, there is huge potential for using microbial factories and bacterial alginate-modifying enzymes to manufacture/modify alginate for high value applications.



Figure 1.3. Alginate applications. Industrial and medical applications are summarised here.

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1.2 *Pseudomonas aeruginosa* and cystic fibrosis

1.2.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram negative rod-shaped bacterium found in soil, water, skin flora and most man-made environments. As a facultative anaerobe, it can utilize a range of carbon sources and alternative terminal electron acceptors under low (no) oxygen conditions. Moreover, its adaptability, numerous nutrient utilization pathways and intrinsic resistance to antimicrobial agents allow it to survive in a wide range of settings. Normally, environmental isolates are non-mucoid (i.e. do not over-produce alginate). However, exposure to stressors including desiccation, osmotic imbalances and antimicrobial agents elicits a stress response and a conversion to a mucoid phenotype (i.e. alginate over-producing state). Although it is generally considered an accidental pathogen to humans, *P. aeruginosa* can cause life-threatening nosocomial infections in the elderly, organ transplant recipients and patients with cancer, severe burns or CF (Lyczak et al. 2000). *P. aeruginosa* lung infections are the leading cause of death in CF patients. Figure 1.4 (page 11) summarizes key virulence factors of *P. aeruginosa* and host lung defenses (Gellatly and Hancock 2013).

For CF lung infections, failure to eradicate *P. aeruginosa* during the acute phase allows it to adapt to the site by over-producing alginate and growing as a biofilm, resulting in chronic infection. Studies on evolution of strains during the course of CF infection (from acute to chronic) show vast variation in phenotypes and genotypes over time (Hogardt et al. 2007; Smith et al. 2006). Often the chronic isolates display reduced inflammatory effects and are less cytotoxic than their acute brethren. This conversion to a chronic state involves mutations in *muca*, *B* or *D* causing mucoid conversion, a loss of flagella, pili and type three secretion systems (Mathee et al. 1999), as well as changes

affecting lipopolysaccharide composition and quorum sensing (Ernst et al. 2007; Winstanley and Fothergill 2009).

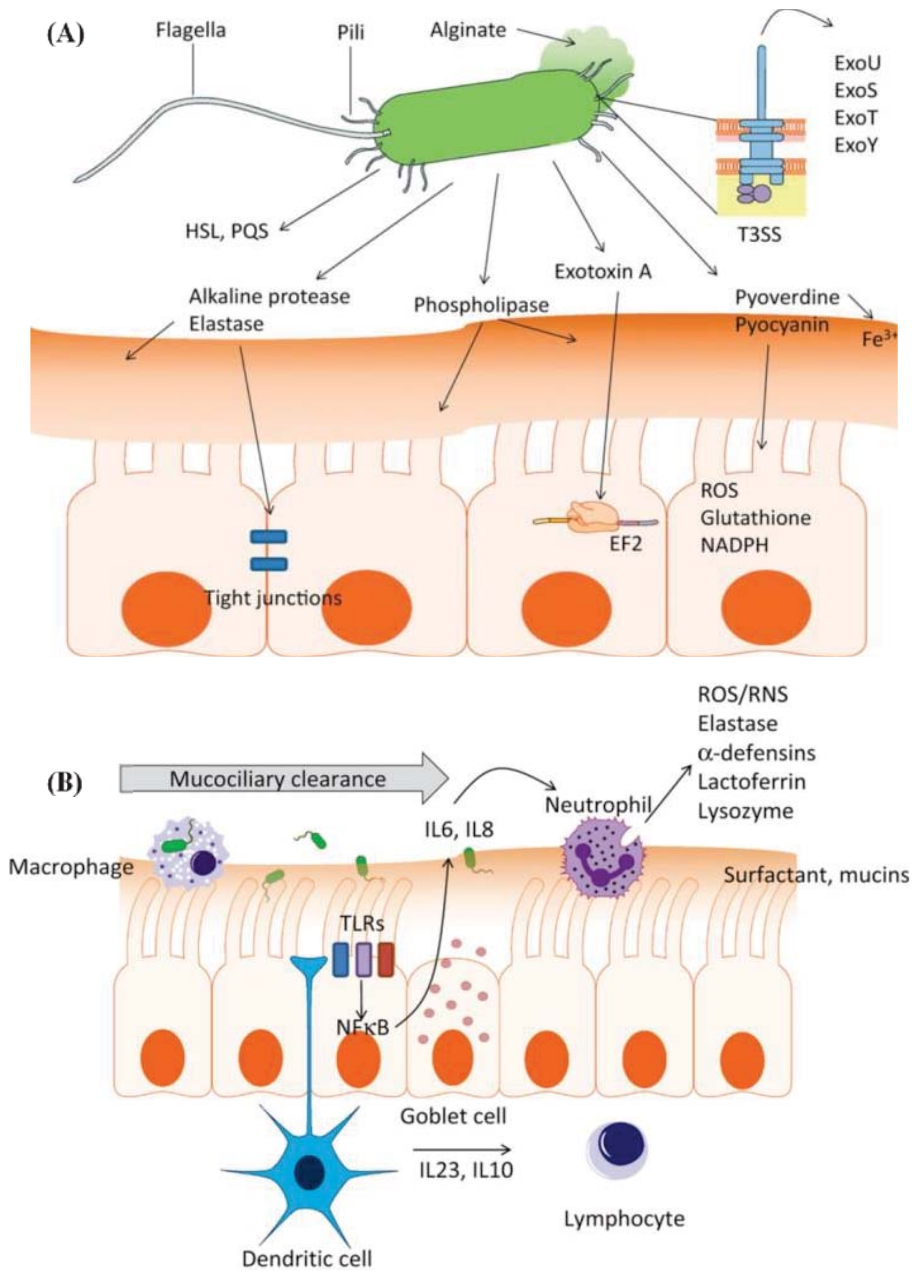


Figure 1.4 *Pseudomonas aeruginosa* vs CF human lung. (A) Virulence factors of *P. aeruginosa* include flagella (motility), type 4 pili (motility, adhesion and aggregation), type 3 secretion system (T3SS, during the acute stage it injects toxins into host cells damaging host membrane integrity), proteases (degradation of host lung surfactant), quorum sensing molecules

(homoserine lactone = HSL and 2-heptyl-3-hydroxy-4-quinolone = PQS), toxins (exotoxin A, pyocyanin), siderophores (pyoverdine), catabolic enzymes (phospholipase, alkaline phosphatase, elastase) and biofilm formation. (B) Host defences include mucociliary clearance, surfactants, antimicrobial peptides (α -defensins, lactoferrin, lysozyme), reactive oxygen (ROS) and nitrogen (RNS) species, release of cytokines and chemokines (NF κ B, IL6, IL8, IL10, IL23), toll-like receptors (TLRs), and various immune cells (macrophage, dendritic cell, goblet cell, lymphocyte, neutrophil). Reuse permission obtained from Oxford University Press (Gellatly and Hancock 2013).

1.2.2 Cystic fibrosis and host defenses

CF patients have mutations in their cystic fibrosis transmembrane conductance gene (CFTR) that encodes a membrane associated ATP-dependent chloride channel. Over 2,000 mutations of this gene have been catalogued in the Cystic Fibrosis Mutation Database (<http://www.genet.sickkids.on.ca/Home.html>). These mutations have been categorized into various classes depending on how severely they impact CFTR expression/translation/localization/function and stability as well as disease manifestations (Marson et al. 2016). Although most mutations can theoretically be rescued by pharmacotherapy and/or gene therapy, this approach is extremely costly and not always reliable. Hence, alternative strategies that target the bacterial infection by *P. aeruginosa* and its ability to produce alginate should be considered.

Mutations of the CFTR gene causes a thickening of epithelial secretions due to imbalanced transport of salt and water transport across cellular membranes (O'Sullivan and Flume 2009). This obstructs the pancreatic duct, intestinal glands and bronchi, preventing normal secretion of digestive enzymes and micro-ciliary clearance from lungs, causing malnutrition and chronic lung infection (Rowe et al. 2005). Manifestations of CF are illustrated in Figure 5A on page 14. Although nutritional

deficiencies can be treated by dietary and enzyme supplements, chronic lung infections are much more serious and difficult to treat. They lead to chronic inflammation, cystic bronchiectasis and severe airflow obstruction, often leading to mortality in patients' thirties (Rowe et al. 2005; Williams et al. 2010).

Normally, the host lungs trap inhaled contaminants in epithelial mucus, sweeping them out by beating of ciliated-epithelial cells (Knight and Holgate 2003). However, thickening of epithelial secretions stalls this clearance mechanism (Knight and Holgate 2003). The host then responds to pathogen colonization by inflammation, secreting antimicrobial peptides (e.g. lysozyme and beta-defensins) to attack the pathogen cell surface (Chronos et al. 2010). Cytokines and chemokines are also produced to recruit and activate innate and adaptive immune responses, including a variety of immune cells (Holt et al. 2008). Particularly, neutrophils play a key role in combating *P. aeruginosa* infections. They phagocytose and kill bacteria, with antimicrobial peptides and reactive oxygen and nitrogen species. However, during chronic CF lung infections, persistent immune stimulation is often counter-productive, causing collateral damage to lung tissues (Williams et al. 2010). This happens because during chronic infections, *P. aeruginosa* overproduces alginate, protecting itself from antibiotics and host defenses, including phagocytosis. This creates an infection that is extremely difficult to treat/eradicate due to the combination of the inflamed host lung environment and the difficult-to-penetrate bacterial biofilm.

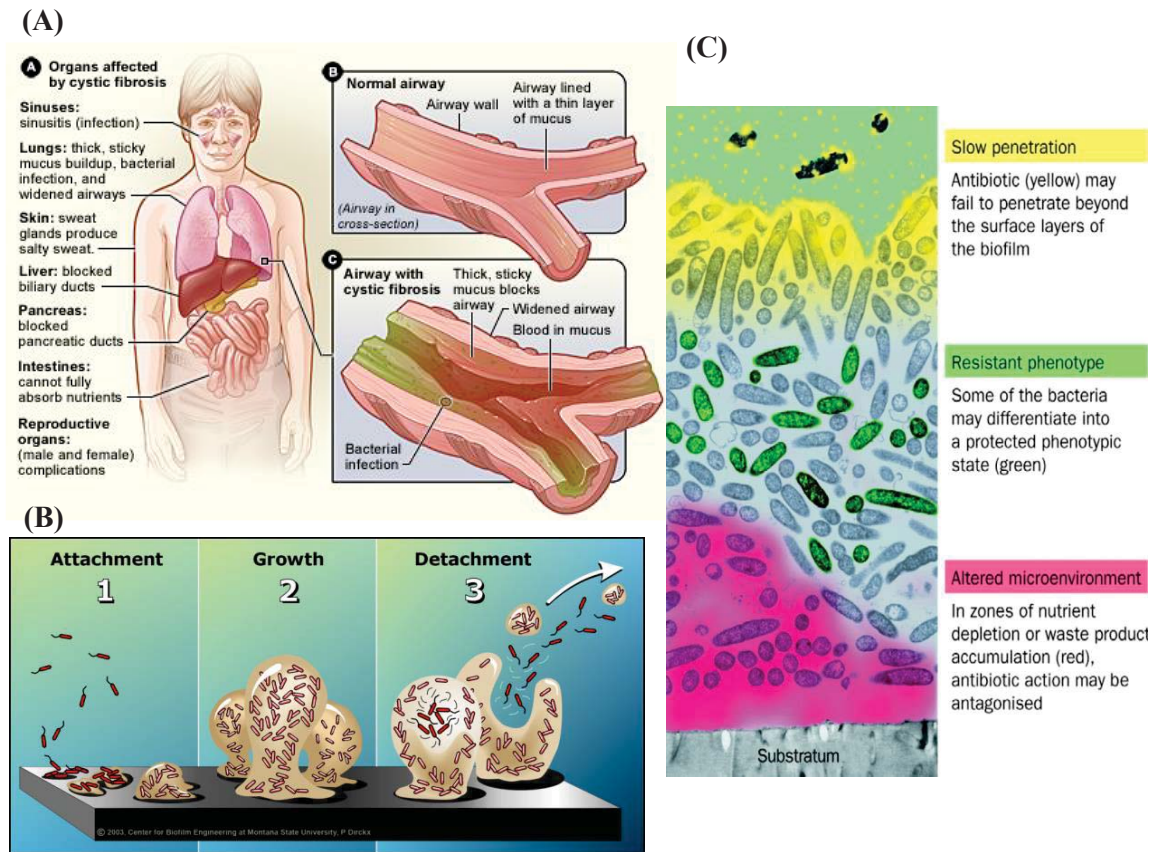


Figure 1.5 Cystic fibrosis, stages of biofilm development and mechanisms of antibiotic resistance. (A) CF affects function of sinus, lungs, skin, liver, pancreas, intestine and reproductive organs. (B) Stages of biofilm formation, attachment, growth and dispersal. (C) Antibiotic resistance mechanisms of *P. aeruginosa* biofilm. Images were reused with permission from (A) National Heart Lung and Blood Institute, (B) Centre for Biofilm Engineering at Montana State University P. Dirckx and (C) Elsevier (Stewart and Costerton 2001).

1.3 Biofilm formation and antimicrobial resistance

A conversion from a free living to biofilm forming state provides cells with distinct advantages including resistance against physical, chemical and biological insults. This conversion is facilitated by complex top-down changes in gene expression controlled by numerous regulatory systems that respond to specific stress-inducing conditions, including predation, starvation, antibiotics, reduced growth rate, dehydration, high osmotic pressure and ionic strength (Devault et al. 1990; Evans and Linker 1973; Govan and Fyfe 1978; Vandevivere and Kirchman 1993).

Biofilm formation has three stages - attachment, growth and dispersal (Figure 1.5B, page 14). During attachment, cells stick to a surface by reversible (electrostatic/vander waals forces) then irreversible interactions via cellular appendages (including type 4 pili, flagella and cup fimbria) and surface-associated polysaccharides (such as Psl and Pel) (Mikkelsen et al. 2011; Wei and Ma 2013). As biofilms mature, they develop into micro-colonies where cells aggregate in a matrix of exopolymeric substances including polysaccharides, proteins, DNA and lipids. In the later stage of dispersal, free living-cells are released by several mechanisms involving mechanical forces, matrix-degrading enzymes and bacteriophages, allowing colonization of new surfaces (Harmsen et al. 2010).

Biofilm growth increases resistance to biocides and antibiotics, posing a major challenge in clinical and industrial settings (Figure 1.5C, page 14) (Stewart and Costerton 2001). Increased resistance occurs by metabolic slowing-down (i.e. reduced oxygen availability and lower growth rate renders certain classes of antibiotics ineffective since they only work on growing/dividing cells) as well as reducing penetration and/or neutralization of antimicrobial agents within the matrix by steep oxygen and chemical gradients. Moreover, resident cells often activate inherent

resistance mechanisms, including reduced membrane permeability, expression of antibiotic degrading enzymes, mutation of antibiotic targets and expression of membrane efflux pumps.

Current widespread use of biocides (in industry and hospitals) and antibiotics (in humans and animals) has encouraged the emergence of highly resistant strains of *P. aeruginosa*, already innately resistant to a wide range of antibiotics, and outbreaks of infection with multi-resistant strains have been reported (Ashish et al. 2012). Thus, alternative strategies should be considered. This study attempts to offer a deeper understanding of alginate production and regulation in the hope of finding better alternatives to counter *P. aeruginosa* biofilms. For instance, matrix degrading enzymes (e.g. alginate lyase) and/or suppression of polysaccharide production (with specific signals) might be an innovative way to better manage *P. aeruginosa* biofilms of clinical and industrial significance.

1.4 Alginate biosynthesis

In previous sections, I touched on the industrial and medical significance of alginate and *P. aeruginosa*. Now I will review current knowledge about bacterial alginate production. Alginate biosynthesis involves four steps: precursor assembly in the cytoplasm, and polymerization, modification and translocation/secretion which are facilitated by a membrane spanning multiprotein complex.

The alginate precursor, guanosine diphosphate (GDP)-mannuronic acid, is synthesized from fructose-6-phosphate in four steps, catalyzed by three enzymes, AlgA, C and D in the cytoplasm (summarized in Figure 1.6, page 18). Fructose-6-phosphate is converted to mannose-6-phosphate (by AlgA), then to mannose-1-phosphate (by AlgC) and GDP-mannose (by AlgA), and finally to GDP-mannuronic acid (by AlgD) (May et al. 1994; Roychoudhury et al. 1989; Shinabarger et al. 1991; Tatnell et al. 1994; Zielinski et al. 1991).

The precursor is polymerized at the inner membrane by Alg8 and Alg44, forming poly-mannuronate (poly-M) which is transported by a multi-protein complex (Alg44, X, G, K) through the periplasm to the outer membrane (OM) protein, AlgE, for secretion (Figure 1.7, page 19). In the periplasm, poly-M is modified by O-acetylation (AlgI, J, F and X) and epimerization (AlgG).

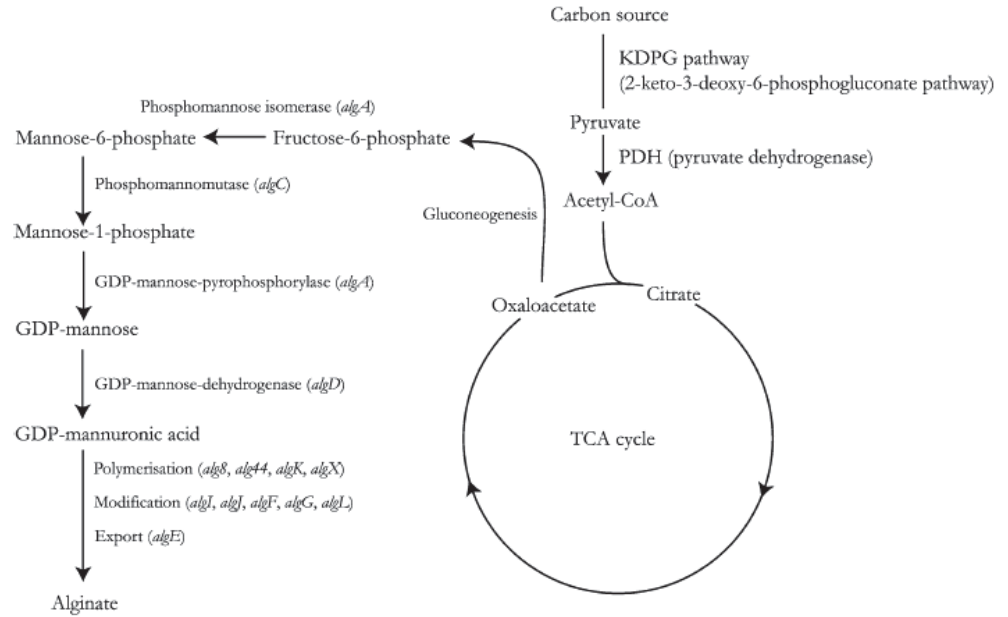


Figure 1.6. A schematic representation of the biochemical pathway for alginate biosynthesis in *P. aeruginosa*. Reuse permission obtained from John Wiley and Sons (Hay et al. 2013).

1.4.1 Polymerization

Polymerization and translocation across the inner membrane involve the inner membrane proteins Alg8 and Alg44 (Moradali et al. 2017; Oglesby et al. 2008; Remminghorst and Rehm 2006a; Remminghorst and Rehm 2006b) (Figure 1.7, page 19). The glycosyltransferase Alg8 transfers the mannuronic acid from the precursor onto a growing poly-M chain while Alg44 is a co-polymerase that aids this activity. The latter has a cytoplasmic PilZ domain that binds to a common bacterial secondary messenger molecule, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Merighi et al. 2007; Whitney et al. 2015). Alg44 also has a periplasmic domain thought to bridge with periplasmic/outer membrane components of the biosynthesis complex

(Oglesby et al. 2008; Remminghorst and Rehm 2006a). In analogous systems (e.g. cellulose biosynthesis), c-di-GMP binding causes local conformational changes that allow activated precursor access to the catalytic site of the glycosyltransferase (Franklin et al. 2011; Morgan et al. 2013; Steiner et al. 2013; Weinhouse et al. 1997; Whitney et al. 2012).

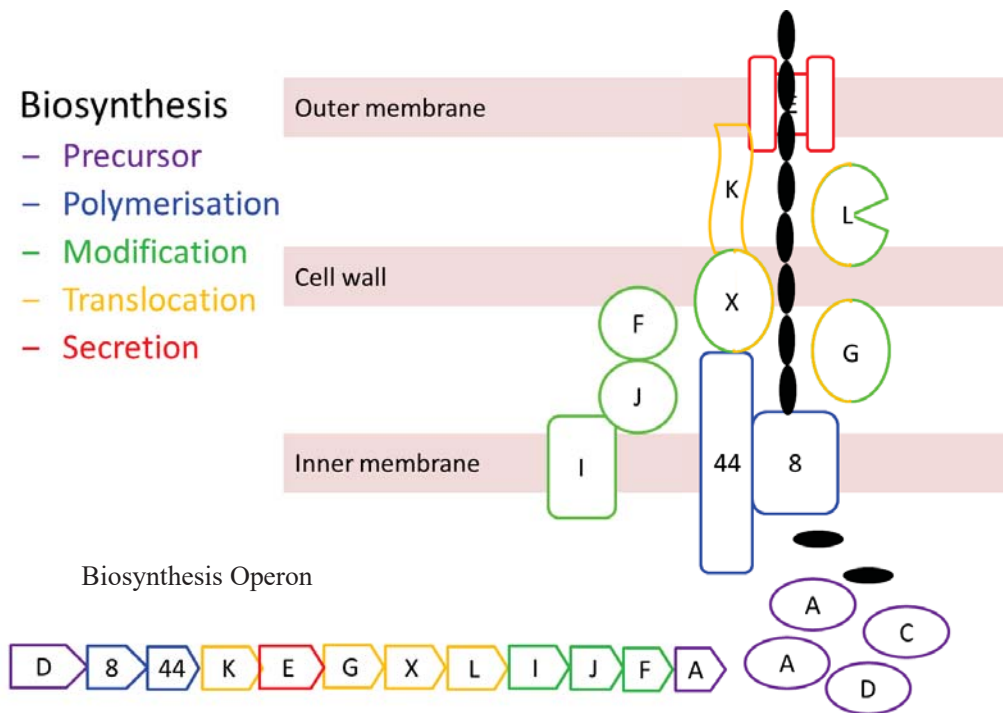


Figure 1.7 A schematic representation of the alginate biosynthesis apparatus and alginate biosynthesis operon. Subunit functions are colour coded. Modified from (Franklin et al. 2011).

1.4.2 Modification

In the periplasm the nascent polymer, poly-M, is O-acetylated by AlgX (and I, J and F) via addition of acetyl groups to O2/O3 positions of M residues (Baker et al. 2014; Franklin et al. 2004; Franklin and Ohman 1996; Franklin and Ohman 2002; Riley et al. 2013) (Figure 1.7, page 19). The polymer is also epimerized by AlgG through conversion of M residues to G via protonation-deprotonation of C5 on the M residue in

the polymer chain (Douthit et al. 2005; Jerga et al. 2006a; Jerga et al. 2006b; Wolfram et al. 2014). These modifications substantially affect alginate material and biological properties. For instance, O-acetylation increases alginate water binding capacity, contributes to biofilm formation by Pseudomonads, and also protects against immune responses and antimicrobial agents (Nivens et al. 2001; Pier et al. 2001; Tielen et al. 2005) while epimerization increases gel forming properties and also contributes to the architecture of *P. aeruginosa* biofilms (Moradali et al. 2015; Morch et al. 2008). O-acetylation also appears to influence the distribution of G residues along the alginate chain (Schurks et al. 2002). While neither enzymatic activity (of AlgX or AlgG) is essential for alginate production, these proteins are thought to form a structural component of the periplasmic scaffold, hence being essential for alginate translocation and secretion (Baker et al. 2014; Gimmestad et al. 2003; Gutsche et al. 2006; Jain et al. 2003; Riley et al. 2013; Robles-Price et al. 2004; Wolfram et al. 2014).

1.4.3 Translocation and secretion

A multi-protein complex is thought to translocate the polymer chain through the periplasm to AlgE for secretion (Figure 1.7, page 19). Several proteins, Alg44, K, G, X and E are thought to contribute to the translocation/secretion scaffold (Gimmestad et al. 2003; Jain et al. 2003; Jain and Ohman 1998; Rehman and Rehm 2013; Rehman et al. 2013; Robles-Price et al. 2004). One of these proteins AlgK, is supposed to help assemble/stabilize the scaffold through its multiple tetratricopeptide-like repeats implicated in protein-protein interactions (Keiski et al. 2010). AlgK is a lipoprotein important for localization of AlgE to the outer membrane, and AlgE is also required for assembly of the complex (Keiski et al. 2010; Rehman and Rehm 2013). Furthermore, through mutual stability and interaction experiments using *alg44*, *K*, *X* and *E* mutants, Rehman et al. (2013) have provided evidence for a membrane spanning multi-protein

apparatus, involved in alginate biosynthesis. Additional experiments have identified an elaborate interaction network between subunits biosynthesis machinery (Moradali et al. 2015). When subunits of the apparatus are missing, the alginate leaks into the periplasm where it is degraded by AlgL, an alginate lyase, that is thought to perform a maintenance role (Bakkevig et al. 2005; Jain and Ohman 2005). However, the necessity of AlgL for alginate production has been controversial. Hence, Chapter 3 was devoted to investigating the biological function of AlgL in alginate production by *P. aeruginosa* (please see Section 1.6 for more about AlgL).

Alginate is secreted through the outer membrane beta barrel porin, AlgE (Hay et al. 2010b; Rehm et al. 1994; Whitney et al. 2011). Structural and molecular dynamics experiments have outlined the conformational landscape for alginate secretion, suggesting that AlgK helps to align the polymer so that it can be threaded through the pore of AlgE (Tan et al. 2014; Whitney et al. 2011).

1.5 Genetics and regulation of alginate production

In the previous section, I reviewed the biosynthesis of alginate. Now, I will give an overview about the genetics and regulation of microbial alginate production. While studies have revealed many intricate details into the signalling pathways regulating alginate biosynthesis, the specific signals perceived by these regulatory sensors remain poorly understood. Nevertheless, some work has shown that alginate production is induced under conditions of desiccation, high osmolarity, sub-inhibitory concentrations of antibiotics and low oxygen conditions (Bragonzi et al. 2005; Chang et al. 2007; Li et al. 2010; Wood and Ohman 2009)

It is widely accepted that alginate over-production provides a hydrated environment that also protects against antimicrobial agents and host defences.

1.5.1 Regulated intramembrane proteolysis cascade

The ‘master switch’ for alginate production in *P. aeruginosa* is the regulatory operon *algU-mucABCD* encoding: AlgU (an alternate sigma factor), its anti-sigma factors MucA and MucB and negative regulators MucC and MucD. This operon responds to envelope stress caused by antimicrobial and oxidizing agents, elevated temperatures, and osmotic imbalances. Mutations in the *muc* genes typically cause a switch to the mucoid phenotype, by unleashing AlgU, driving expression of its own operon, several other genes involved in alginate regulation as well as the alginate biosynthesis gene cluster (Firoved et al. 2002; Hay et al. 2014; Wozniak et al. 2003). Mutation of anti-sigma factor MucA forces the system into a ‘permanent on state’ (Hay et al. 2014) (Figure 1.8C, page 23). Normally, in the ‘off state’ (i.e. low stress) AlgU is sequestered at the inner membrane (Figure 1.8A, page 23) by MucA-MucB (Cezairliyan and Sauer 2009; Wood and Ohman 2009).

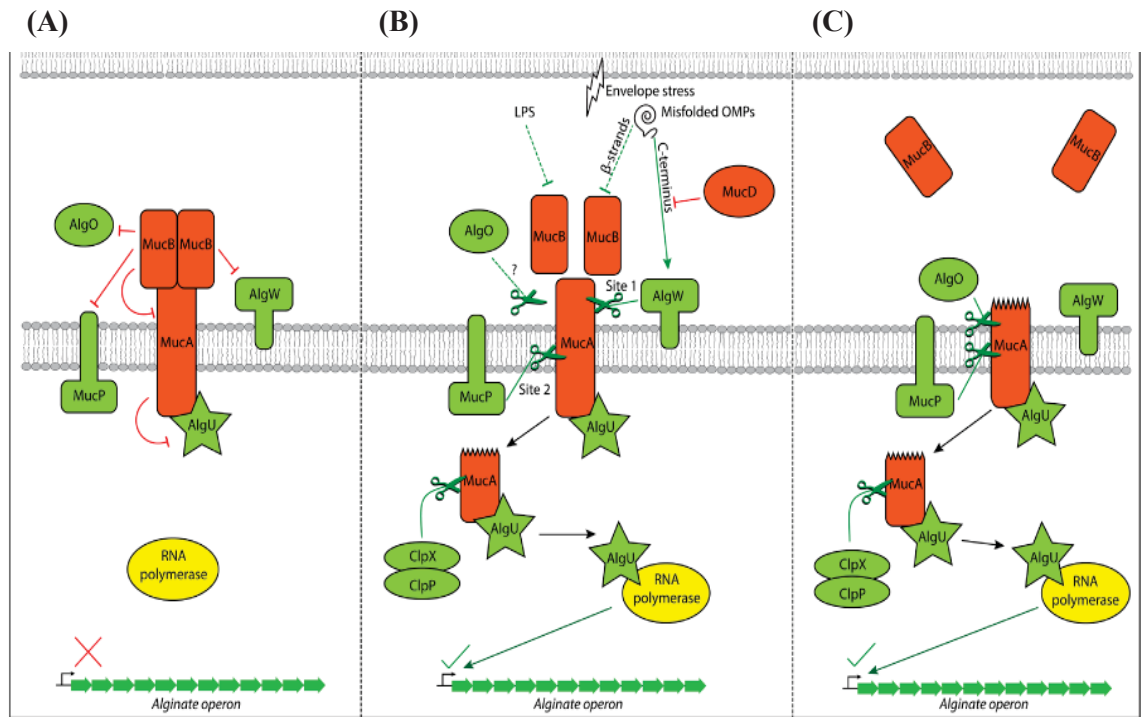


Figure 1.8 The ‘switch’ for alginate production. Proteins and lines shown in green have a positive effect on alginate production; proteins and lines shown in red have a negative effect on alginate production. Dotted lines indicate an unknown/unclear mechanism. **(A)** Off state. The switch is turned OFF in the absence of envelope stress. In this state, AlgU is sequestered at the inner membrane by MucA–MucB, protecting MucA from proteolysis by AlgW and MucP. **(B)** Induced On state. Envelope stress (thunderbolt) turns ON the switch. Mislocalized/misfolded cellular components activate AlgW destabilizing the MucA–MucB interaction. MucD degrades and/or repairs damaged OMPs that activate the cascade. Cleavage of MucA by AlgW is followed by site-2 proteolysis by MucP, releasing the cytoplasmic domain of MucA with AlgU bound into the cytosol where ClpXP degrade MucA, freeing AlgU to interact with RNA polymerase and drive expression of alginate production genes. **(C)** Permanent On state. Example of a clinical *mucA* mutant (*mucA22*) with a truncated MucA is no longer able to seek MucB for protection, rendering it susceptible to proteolysis by AlgO and possibly other proteases. Reuse permission obtained from John Wiley and Sons (Hay et al. 2014).

In response to envelope stress, the system is switched 'on'; AlgU is released from its anti-sigma factor complex by a regulated intramembrane proteolysis (RIP) cascade involving various proteases (Figure 1.8B, page 23). Envelope stress causes mis-folding and mis-localization of membrane components which disrupt the MucA-MucB interaction and activate protease AlgW, cleaving MucA. (Cezairliyan and Sauer 2009; Chaba et al. 2011; Kulp and Kuehn 2011; Lima et al. 2013; Qiu et al. 2007; Wood and Ohman 2009). MucA is further hydrolyzed by MucP (Qiu et al. 2007), releasing truncated MucA still bound to AlgU into the cytosol where it is rapidly degraded by ATP-dependent cytoplasmic proteases, freeing AlgU to drive expression of alginate biosynthesis genes (Qiu et al. 2008b).

MucD negatively regulates the RIP cascade by chaperoning and/or degrading misfolded proteins that would otherwise activate AlgW or MucP proteases (Damron and Yu 2011; Qiu et al. 2007; Yorgey et al. 2001) (Figure 1.8B, page 23). Intriguingly, MucD also interacts with a component of the alginate biosynthetic machinery, AlgX (Gutsche et al. 2006; Hay et al. 2012); however, the significance of this interaction is unknown. In clinical *P. aeruginosa* strains from CF lung infections, the alginate operon is in a 'permanent on state' (Figure 1.8C, page 23). This short-circuiting occurs by mutations truncating MucA, preventing its interaction with MucB and exposing it to degradation by alternative proteases such as AlgO (Qiu et al. 2007; Reiling et al. 2005). The desiccating environment of CF lungs is thought to elicit this switch, first by the induced and then by the permanent on state.

1.5.2 Transcriptional regulation

Alginate production is regulated transcriptionally by sigma factors, two component signal transduction systems (TCST) and other DNA binding proteins (Figure 1.9, page 26). For AlgU to drive alginate production, other sigma factors are sequestered by their

cognate anti-sigma factors (Pineda et al. 2004; Yin et al. 2013; Yuan et al. 2008). Certain sigma factors such as RpoN reduce alginate production under nitrogen rich conditions (Boucher et al. 2000).

At least 2 TCST systems are involved in regulating alginate production: KinB-AlgB and FimS-AlgR (Figure 1.9A, page 26). Generally, TCST systems have a sensor kinase (SK) (e.g. KinB and FimS) that autophosphorylates itself in response to environmental signals, and activates its cognate response regulator (RR) via phospho-transfer. However, the exact signals of the SKs systems are unknown. Moreover, both these systems behave non-canonically - RRs can enhance alginate production independently of their SKs and phosphorylation (Ma et al. 1998; Wozniak and Ohman 1993). Also KinB could be a phosphatase that is involved in AlgW-mediated degradation of the MucA (Chand et al. 2012; Damron et al. 2009) (Figure 1.9A-D, page 26).

Numerous DNA-binding proteins control expression from *PalgD* promoter (Baynham et al. 2006; Kato et al. 1990)(Figure 1.9B-C, page 26). These include the global regulators AlgU and AmrZ (Jones et al. 2013; Pryor et al. 2012; Tart et al. 2006; Waligora et al. 2010), a histone-like DNA-binding protein, AlgP that enhances alginate production in response to nitrogen availability (Deretic and Konyecsni 1990) an integration host factor like heterodimer (IHF α and IHF β) (DelicAttree et al. 1996), and various other transcription factors including CysB and Vfr (DelicAttree et al. 1997) (Figure 1.9C, page 26).

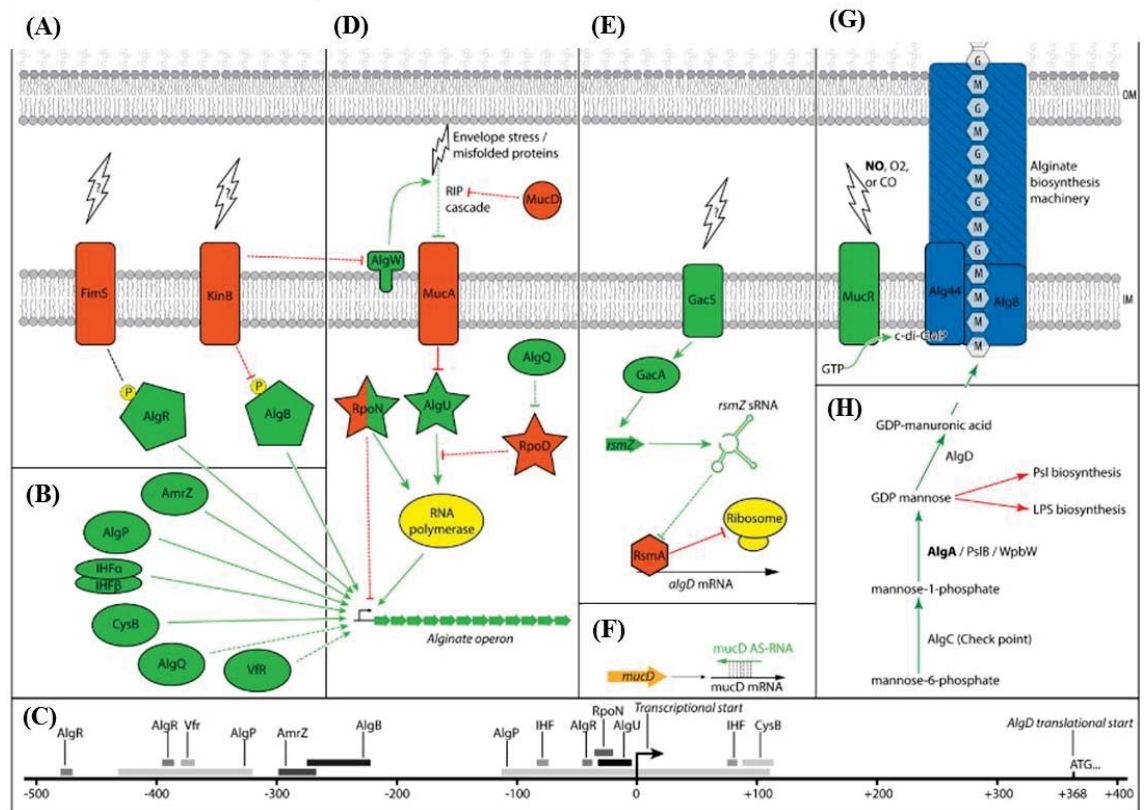


Figure 1.9 Schematic representation of various regulatory mechanisms of alginate biosynthesis. Green proteins and lines have a positive effect on alginate production; red proteins and lines have a negative effect on alginate production. Dotted lines indicate an unknown/unclear mechanism. **(A)** Two-component signal transduction systems, FimS/AlgR and KinB/AlgB. **(B)** Transcriptional regulation through DNA-binding proteins. **(C)** Schematic map of the approximate binding sites of various transcriptional regulators on the *P. aeruginosa* *algD* promoter. **(D)** Sigma/anti-sigma factors. **(E)** Posttranscriptional regulation through the Gac/Rsm sRNA system in *Azotobacter vinelandii*. **(F)** Posttranscriptional regulation through a natural antisense transcript (MucD-AS) that promotes alginate production by blocking the translation of *mucD* mRNA. **(G)** Posttranslational regulation by c-di-GMP. MucR synthesizes a pool of c-di-GMP near the alginate co-polymerase Alg44. Binding of c-di-GMP to Alg44 is essential for alginate biosynthesis. **(H)** Posttranscriptional regulation by substrate competition. Reuse permission obtained from John Wiley and Sons (Hay et al. 2014).

1.5.3 Post-transcriptional regulation

Mechanisms of post-transcriptional control of alginate production are shown in Figure 1.9E-F, page 26). In *A. vinelandii*, the central TCST system, GacS-GacA, activates expression of noncoding small RNAs which sequester the translational regulatory protein, RsmA - preventing it from binding and repressing translation from the *algD* mRNA transcript (Manzo et al. 2011). In *P. aeruginosa* a natural antisense transcript (*mucD*-AS), when overexpressed, significantly induces alginate production and biofilm formation by blocking MucD production (Yang et al. 2011b).

1.5.4 Post-translational regulation

Mechanisms of post-translational control of alginate production are shown in Figure 1.9G-H. Substrate competition for GDP-mannose by alginate, Psl and B-band LPS biosynthesis pathways may indirectly affect alginate yields (Byrd et al. 2009; Shinabarger et al. 1991). Additionally, the generic bacterial secondary messenger, c-di-GMP, drives alginate production post-translationally by binding to the PilZ domain of Alg44, the alginate co-polymerase (Merighi et al. 2007; Whitney et al. 2015). Another inner membrane protein, MucR, involved in c-di-GMP turnover is thought to impart a localised pool of c-di-GMP (Hay et al. 2009b)(Figure 1.9G, page 26). However, it is unknown how its activity is regulated nor is it known what signal it perceives. Thus, the role of MucR was studied in detail in Chapter 4 (please see Section 1.7 for more about MucR).

As mentioned earlier, two major gaps provide the foci of this study. AlgL, an alginate lyase, and MucR, a sensor protein with c-di-GMP turnover activity (discussed in more detail below).

1.6 Function of AlgL in alginate production

As reviewed in Section 1.4, when components of the alginate biosynthesis apparatus are missing, alginate is thought to leak into the periplasm, where it is degraded by alginate lyases (e.g. AlgL), releasing free uronic acid oligomers. AlgL preferentially cleaves the non-acetylated alginate chain via beta elimination, releasing dimeric and/or trimeric alginate oligosaccharides with 4-deoxy-alpha-L-erythro-hex-4-enuronosyl groups at their non-reducing ends and beta-D-mannuronate at their reducing end (Farrell and Tipton 2012). Since mature alginate is O-acetylated, such activity is consistent with a maintenance role, i.e. degrading misguided alginate from the periplasm. However, because of the inconsistencies in the literature (outlined below), it has been difficult to pinpoint the exact function of AlgL. Major inconsistencies revolve around its requirement for alginate production. While several studies support the requirement of AlgL for alginate biosynthesis, others suggest otherwise (Albrecht and Schiller 2005; Bakkevig et al. 2005; Boyd et al. 1993; Jain and Ohman 2005; Monday and Schiller 1996; PenalozaVazquez et al. 1997; Trujillo-Roldan et al. 2003).

Boyd et al. (1994) and PenalozaVazquez et al. (1997) demonstrated that expression of *algA* (an essential downstream gene) in transposon-generated polar *algL* mutants in *P. aeruginosa* and *P. syringae* restored alginate production on solid media, and Trujillo-Roldan et al. (2003) showed that a non-polar *algL* deletion mutant in *A. vinelandii* could still produce alginate. In contrast, other studies support a requirement for AlgL in alginate production. For instance, alginate yield in a polar *algX* mutant of the clinical isolate, *P. aeruginosa* FRD1, could only be restored by plasmid-borne expression of *algX* together with downstream genes *algL* and *algA* (Monday and Schiller 1996). Similarly, Bakkevig et al. (2005) showed that expression of only *algC* (involved in alginate precursor production) in an *algC algL* double mutant (generated in

Pseudomonas fluorescens) did not restore alginate production. Jain & Ohman (2005) replaced the *PalgD* promoter of *P. aeruginosa* FRD1 with an inducible one prior to swapping out the *algL* gene with a gentamicin resistance marker and revealed that inducing alginate production in the absence of AlgL during planktonic growth led to an accumulation of polymeric substances in the periplasm, eventuating in cell lysis. A later study reached a similar conclusion for cells grown in liquid media, further emphasising the need for AlgL lyase activity in alginate biosynthesis (Albrecht & Schiller 2005).

The above inconsistencies may relate to differences in experimental setup (parent species/strain and mutant generation) and technical challenges. For example, stabilization of alginate production in parent strains by chemical mutagenesis (Bakkevig et al. 2005; Boyd et al. 1993; Darzins et al. 1986; Wang et al. 1987), the use of uncharacterized plasmids (PenalozaVazquez et al. 1997) or placing alginate biosynthesis genes under control of inducible promoters (Albrecht and Schiller 2005; Bakkevig et al. 2005; Jain and Ohman 2005). These approaches introduced unknown genetic changes and deregulated alginate production from normal cellular regulation. Moreover, the methods used for generating *algL* mutants - transposon- and marker-based strategies – often led to technical challenges in complementation due to polar effects (Albrecht and Schiller 2005; Boyd et al. 1993; Jain and Ohman 2005; Monday and Schiller 1996; PenalozaVazquez et al. 1997; Trujillo-Roldan et al. 2003).

Despite these issues, two preliminary models have been proposed for the role of AlgL in alginate production (Figure 1.10, page 31). While both models assign AlgL a maintenance role to degrade misguided alginate in the periplasm – to partner the previous biochemical studies (Farrell and Tipton 2012), they disagree on whether it is a subunit of the complex or a free periplasmic protein. Bakkevig et al. (2005) propose that AlgL only plays a maintenance role (Figure 1.10A, page 31), and hence could be a free

periplasmic protein. However, Jain & Ohman (2005) suggest that AlgL is also involved in secretion/translocation of the polymer (Figure 1.10B, page 31), and thus should be a structural component of the complex. However, direct evidence of AlgL interacting with the apparatus is still lacking. Furthermore, if and how AlgL influences alginate polymer length and size distribution is poorly understood.

Although alginate is considered a major component of mucoid *P. aeruginosa* biofilms, its function in attachment remains unclear. So far, whether alginate-degrading enzymes contribute to cell dispersal remains highly controversial. For example, some researchers postulate that alginate activity (supplemented extracellularly) could facilitate dispersal, increasing susceptibility to antimicrobial agents (Alkawash et al. 2006). However, findings from other researchers suggest otherwise (Germoni et al. 2016; Lamppa and Griswold 2013). Moreover, while over-expression of AlgL increased sloughing from biofilms grown on solid plates (Boyd and Chakrabarty 1994), it is not known how deletion of *algL* could affect biofilm formation. While other researchers have shown that *P. aeruginosa* produces PslG, which degrades Psl polysaccharide, leading to biofilm disassembly (Yu et al. 2015), it is presently unknown if endogenously produced alginate lyase participates in cell release from *P. aeruginosa* biofilms.

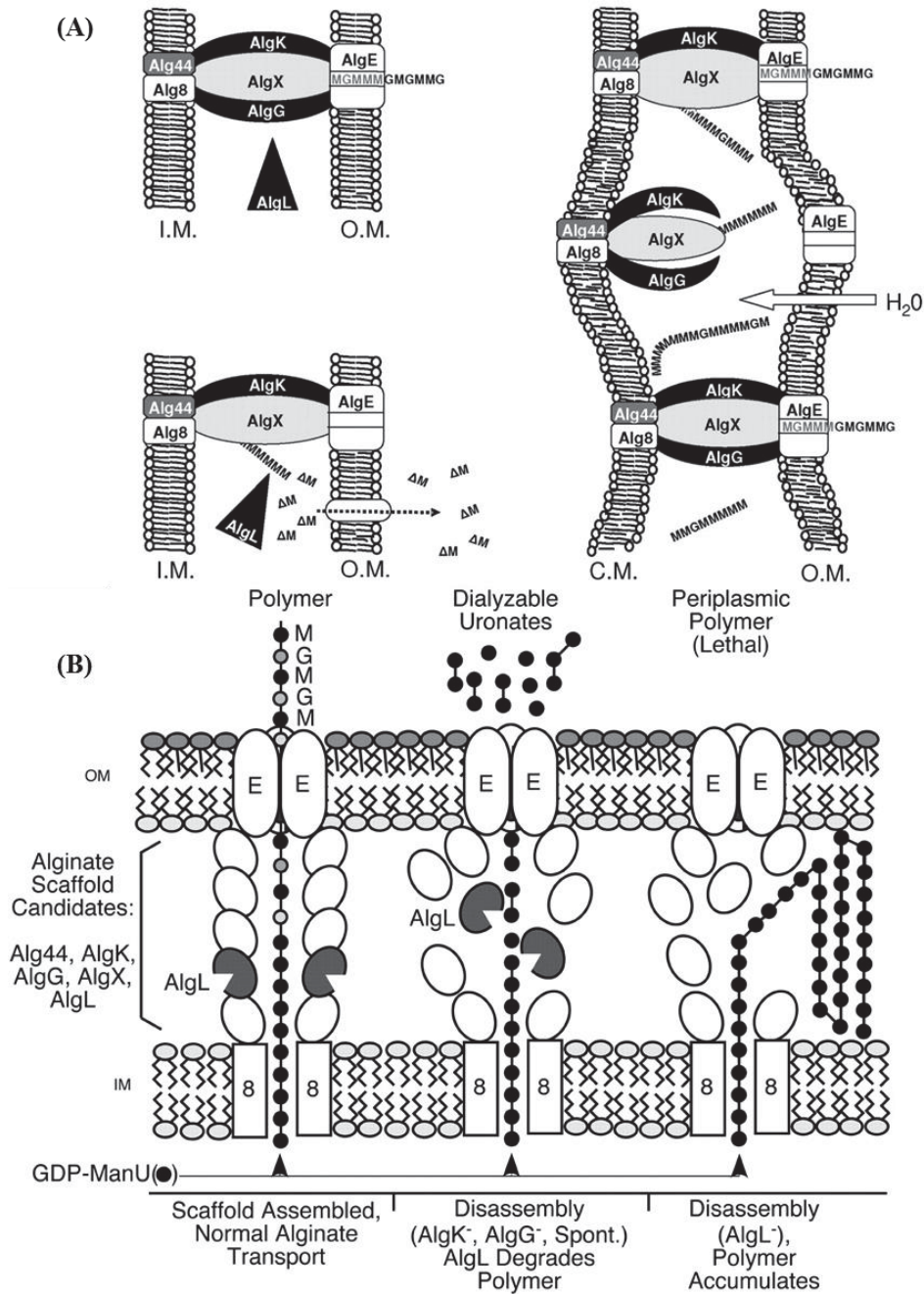


Figure 1.10 Preliminary models for the role of AlgL in alginate biosynthesis. In both models, AlgL plays a maintenance role, degrading misguided alginate from the periplasm. (A) In one model proposed by Bakkevig et al. (2005), AlgL is a free periplasmic protein. (B) In another model proposed by Jain & Ohman (2005), AlgL is a structural component of the complex. Images were reused with permission from the American Society of Microbiology (Bakkevig et al. 2005; Jain & Ohman 2005).

1.7 Function of MucR in regulating alginate production

MucR is thought to impart a localised pool of c-di-GMP, driving alginate production post-translationally (Hay et al. 2009b) (Figure 1.11, page 33). It has an inner membrane MHYT sensor domain, and cytoplasmic GGDEF and EAL domains that make and break c-di-GMP via diguanylate cyclase (DGC) and phosphodiesterase (PDE) activity, respectively (Hay et al., 2009b; Li et al. 2013). Li et al. (2013) suggest that the c-di-GMP turnover function of MucR is growth-mode dependent, and that it may also be involved in NO- and glutamate-induced dispersal. However, what exact signal is perceived by MucR and how signal perception affects alginate production specifically are still poorly understood.

The sensor domain of MucR has three MHYT motifs (named after its amino acid sequence: Methionine, Histidine, Tyrosine, Threonine) predicted to co-ordinate a copper ion for perceiving a diatomic gas – such as nitric oxide (NO) (Galperin et al. 2001; Hay et al. 2009b). Nitric oxide and other nitro-active intermediates are produced by *P. aeruginosa* during denitrification which occurs under microaerophilic conditions, commonly encountered during biofilm growth, whereby the organism utilises nitrate as an alternative electron acceptor. Denitrification intermediates are linked to reduced alginate production (Wood et al. 2007; Zumft 1997).

However, whether nitrate, the initial substrate for denitrification (or other signals), suppresses alginate biosynthesis in a manner dependent on MucR's sensor (and output) domains requires further elucidation.

Since most enzymes participating in c-di-GMP turnover form multimeric quaternary structures—dimers or tetramers (Barends et al. 2009; De et al. 2009; Paul et al. 2007; Phippen et al. 2014; Romling et al. 2013; Sharma et al. 2014; Tarutina et al. 2006;

Tchigvintsev et al. 2010) — the activity of MucR could be dictated by its oligomeric state in response to its putative signal(s). However, it is unclear if (and how) MucR and its signal control alginate production. In particular, it is unknown if this pathway is specifically dependent on c-di-GMP at a post-translational and/or transcriptional level. A loss of alginate production enhances swarming motility and surface attachment and increases production of Pel and Psl exopolysaccharides (Ghafoor et al. 2011; Hay et al. 2009b). It is thus proposed that if the putative signal perceived by MucR suppresses alginate production, then motility, attachment, and Pel and Psl production should increase. Since Pel and Psl are involved in cell-cell and cell-surface interactions, the effects of MucR and its signal may also affect biofilm characteristics, such as its thickness, compactness and cell survival.

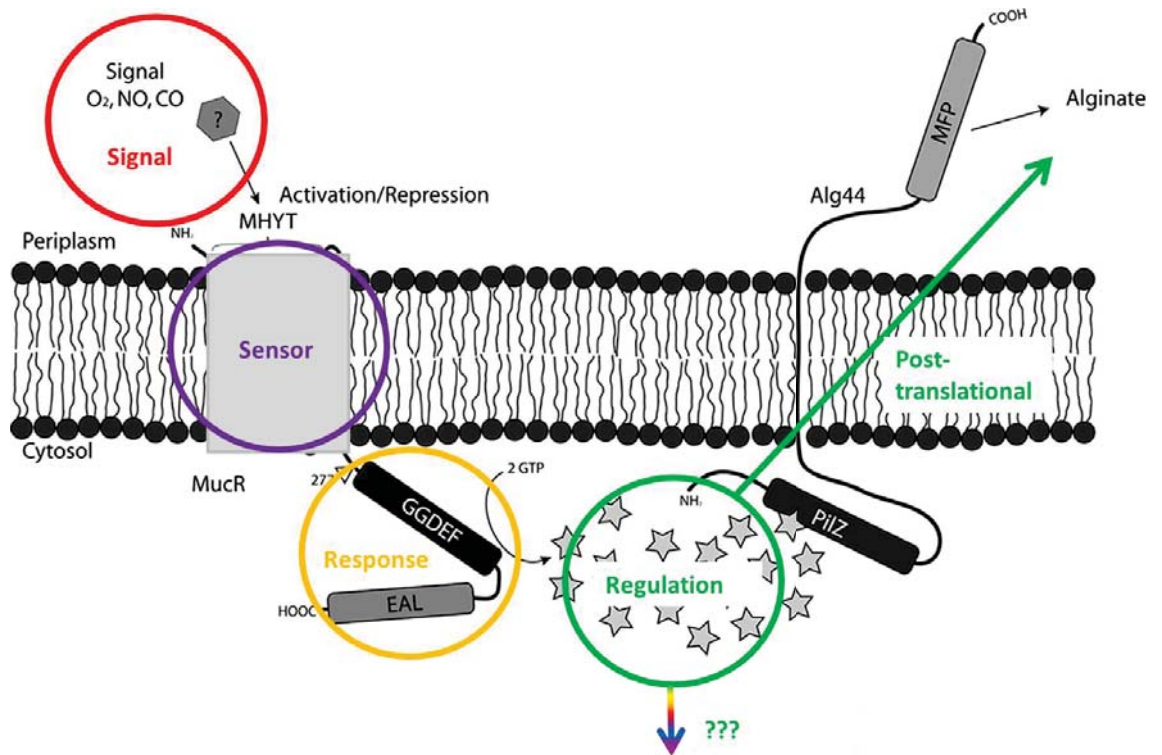


Figure 1.11 Proposed model for the role of MucR in alginate biosynthesis. A putative signal is perceived by the MHYT sensor domain of MucR, this in turn affects the activity of its output

domain which is involved in c-di-GMP synthesis (GGDEF) and degradation (EAL). This affects the concentration of intracellular c-di-GMP levels which activate alginate production by binding to the PilZ domain of Alg44, the alginate co-polymerase. It is unclear if the c-di-GMP pool imparted by MucR also impacts alginate production transcriptionally, nor is it known if the MucR-signal pathway influences other phenotypes in a c-di-GMP dependent manner. Reused and modified with permission from the American Society of Microbiology (Hay et al. 2009).

1.8 Aims and scientific questions

Based on our current knowledge on alginate production in bacteria outlined above, I proposed to work on two proteins, AlgL and MucR, that are considered to be involved in alginate production. I designed and carried out a series of experiments (see Chapter Two) to attempt to answer the following questions using *P. aeruginosa* as a model organism:

1. What role do AlgL and its lyase activity play in alginate production, polymer length control and composition?
2. Is AlgL a free periplasmic protein or a subunit of the biosynthesis complex?
3. What role does AlgL play in biofilm attachment and dispersal?
4. Are both sensor and output domains of MucR important for alginate biosynthesis?
5. Is nitrate the putative signal perceived by MucR?
6. Does MucR-nitrate pathway regulate alginate production at post-translational/transcriptional level through c-di-GMP?

In addition, I also explored the broader implications of MucR-nitrate pathway on the biofilm lifestyle including swarming, attachment, expression of *Psl* and *Pel* biosynthesis genes and biofilm thickness, compactness and survival.

The new information generated from the current study has contributed to the better understanding of the functions of these two proteins. My findings in conjunction with others' research, could help contribute to the development of measures for disease treatment and bacterial production of alginate in the future.

2. Chapter Two: Materials and Methods

In Chapter 2, I outline and illustrate both general and specific materials and methods used for this study.

2.1 Strains, plasmids and oligonucleotides

Tables 2.1, 2.2 and 2.3 (pages 36-42) list the bacterial strains, plasmids and oligonucleotides used in the present study. All oligonucleotides were synthesised by Integrated DNA Technologies Ltd, USA.

TABLE 2.1 Bacterial strains used in present study

Strains	Description	Source
<i>P. aeruginosa</i>		
PDO300	<i>mucA22</i> mutant derived from PAO1. Encodes truncated MucA, inducing alginate over-production	(Mathee et al. 1999)
PDO300 Δ <i>mucR</i>	<i>mucR</i> marker-free deletion mutant derived from PDO300	(Hay et al. 2009b)
PDO300 Δ <i>algL</i>	<i>algL</i> marker-free deletion mutant derived from PDO300	This study
PDO300 Δ <i>alg44</i>	<i>alg44</i> marker-free deletion mutant derived from PDO300	(Remminghorst and Rehm 2006a)
PDO300 Δ <i>algK</i>	<i>algK</i> marker-free deletion mutant derived from PDO300	(Rehman et al. 2013)
PDO300 Δ <i>algX</i>	<i>algX</i> marker-free deletion mutant derived from PDO300	(Gutsche et al. 2006)
PDO300 Δ <i>algE</i>	<i>algE</i> marker-free deletion mutant derived from PDO300	(Hay et al. 2010b)

E. coli

TOP10	<i>E. coli</i> cloning strain	Invitrogen
S17-1	thi-1 proA hsdR17 (rK- mK- recA1; tra gene of plasmid RP4 integrated in chromosome	(Simon et al. 1983)

TABLE 2.2 Plasmids used in this study

Plasmids	Description	Source
pBBR1MCS-5	Gentamicin resistance, broad-host-range vector, <i>lacZ</i> promoter	(Kovach et al. 1995)
pBBR1MCS-5: <i>mucR</i>	KpnI-ClaI <i>mucR</i> fragment inserted into pBBR1MCS-5	(Hay et al. 2009b)
pBBR1MCS-5: <i>mucR</i> MHYT I	<i>mucR</i> with 1st MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i> MHYT II	<i>mucR</i> with 2nd MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i> MHYT III	<i>mucR</i> with 3rd MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i> MHYT I, II	<i>mucR</i> with 1st & 2nd MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i> MHYT I, III	<i>mucR</i> with 1st & 3rd MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i> MHYT II, III	<i>mucR</i> with 2nd & 3rd MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i> MHYT I, II, III	<i>mucR</i> with 1st, 2nd & 3rd MHYT motif to MAYT in pBBR1MCS-5	Rehm Lab
pBBR1MCS-5: <i>mucR</i>	<i>mucR</i> with GGDEF motif to GGAAF in	Rehm Lab

GGDEF	pBBR1MCS-5	
pBBR1MCS-5: <i>mucR</i>	<i>mucR</i> with EAL motif mutated to AAL in	Rehm Lab
EAL	pBBR1MCS-5	
pEX100TΔ <i>mucR</i> ΩGm	Ampicillin, carbenicillin and gentamicin resistance, pEX100T with SmaI-inserted <i>mucR</i> deletion construct	(Hay et al. 2009b)
pFLP2	Ampicillin and carbenicillin resistance, broad-host-range vector encoding Flp recombinase	(Hoang et al. 1998)
mini-CTX- <i>lacZ</i> - <i>pelA</i> _{wt}	Tetracycline resistance, vector miniCTX- <i>lacZ</i> with BamHI–EcoRI inserted c-di-GMP sensitive promoter	(Baraquet et al. 2012)
mini-CTX <i>P</i> <i>algL</i> <i>Z</i>	Tetracycline resistance, vector miniCTX- <i>lacZ</i> with HindIII–BamHI inserted <i>algD</i> promoter region	(Hay et al. 2012)
pTZ110: <i>Ppsl</i>	Carbenicillin resistance, vector pTZ110 with HindIII–BamHI inserted <i>pslA</i> promoter region	(Overhage et al. 2005)
pTZ110: <i>Ppel</i>	Carbenicillin resistance, vector pTZ110 with EcoRI–BamHI inserted <i>pelA</i> promoter region	(Ghafoor et al. 2011)
pBBR1MCS-5: <i>wspR</i>	<i>wspR</i> gene encoding a highly active DGC, WspR, inserted into vector pBBR1MCS-5	(Hay et al. 2009b)
pEX100TΔ <i>algL</i> ΩGm	Ampicillin, carbenicillin and gentamicin resistance, vector pEX100T with SmaI-inserted <i>algL</i> deletion construct	Rehm Lab
pHERD20T	Ampicillin and carbenicillin resistance, vector with arabinose inducible promoter (araC- <i>P</i> _{BAD} cassette)	(Qiu et al. 2008a)
pHERD20T: <i>algL</i>	<i>algL</i> ORF (NcoI-SmaI) in pHERD20T	This study

pHERD20T: <i>algL</i> _{H202A}	<i>algL</i> ORF with H202A mutation (NcoI-SmaI) in pHERD20T	This study
pHERD20T: <i>algLx6his</i>	<i>algL</i> ORF with C-terminal hexahistidine tag (NcoI-SmaI) in pHERD20T	This study
pBBR1MCS-5: <i>algX</i>	<i>algX</i> ORF in pBBR1MCS-5	(Moradali et al. 2015)
pBBR1MCS-5: <i>algXS269A</i>	<i>algX</i> ORF in pBBR1MCS-5, with catalytic serine 269 mutated to alanine	(Moradali et al. 2015)

TABLE 2.3 Oligonucleotides used in present study

Oligonucleotides	Sequence	Source
Complementation of <i>mucR</i> mutant		
MucRN(KpSDNd)	AGCAAGGTACCAGGAGACGCTCATATGCTTA TCAGCA	(Hay et al. 2009b)
MucR(Cla)	GAGTAATCCATAAATCAGG	(Hay et al. 2009b)
Mutation of MHYT I to MAYT I		
MHYT_1_F	GGCATGCTCGCCTTCAGCCTGC	Rehm Lab
MHYT_1_R	GATGCCGAAGCCCATGGCGAAA	Rehm Lab
MHYT_H59A_F	TGGTCGATGGCCTTCGTCGGCATGCTCGCCTT CAGCCTGC	Rehm Lab
MHYT_H59A_R	GACGAAGGCCATCGACCAGATGCCGAAGCCC ATGGCGAAA	Rehm Lab

Mutation of MHYT II to MAYT II

MHYT_2_F	GGGATGGCCGCCCTGCTGATGA	Rehm Lab
MHYT_2_R	GATGCCGCTGCCCATCAGCAGG	Rehm Lab
MHYT_H122A_F	GCCGCCATGGCCTACACCGGGATGGCCGCCCT GCTGATGA	Rehm Lab
MHYT_H122A_R	GGTGTAGGCCATGGCGGCGATGCCGCTGCCC ATCAGCAGG	Rehm Lab

Mutation of MHYT III to MAYT III

MHYT_3_F	GGGATGGCCGCCGCGCAGTTCC	Rehm Lab
MHYT_3_R	GATGGCGCAGCCCATCACCAGG	Rehm Lab
MHYT_H188A_F	GTCGGCATGGCCTACACCGGGATGGCCGCCG CGCAGTTCC	Rehm Lab
MHYT_H188A_R	GGTGTAGGCCATGCCGACGATGGCGCAGCCC ATCACCAGG	Rehm Lab

GGDEF to GGAAF mutation

GGDEF_F	CTGCTGATCGAGGCCCGAGAGC	Rehm Lab
GGDEF_R	GAGGCGGGCGATGGTGTCTCTGG	Rehm Lab
GGDEF_DE344- 345AA_F	GGCGGCGCCGCGTTCGTCCTGCTGATCGAGGC CCGAGAGC	Rehm Lab
GGDEF_DE344- 345AA_R	GACGAACGCGGCCCGCCGAGGCGGGCGATG GTGTCCTGG	Rehm Lab

Mutation of EAL to AAL mutation

EAL_F	CTGCGCTGGGAGCATCCGCAAC	Rehm Lab
EAL_R	CATCGGTCCGTTCGGCGCCAGT	Rehm Lab
EAL_E469A_F	ATCGGCGTGGCCGCGCTGCTGCGCTGGGAGC ATCCGCAAC	Rehm Lab
EAL_E469A_R	CAGCGCGGCCACGCCGATCATCGGTCCGTTCG GCGCCAGT	Rehm Lab

Confirming *mucR* knockout

MucR upXout	GACTCGTGCAATAATATCAGGTTG	(Hay et al. 2009b)
MucR downXout	GCATGTTTTCTCGTTATTGGATAGA	(Hay et al. 2009b)

Generating c-di-GMP sensitive promoter

pPel _{fullfor}	CGGCGAATTCCTGGTGCGGTTCCCTCGCACGCA AC	(Baraquet et al. 2012)
pPel _{fullrev}	GATCGGATCCACGGCGATTCCCTTCTTGCTG	(Baraquet et al. 2012)

Confirm integration of miniCTX based vectors

Pser Up	CGAGTGGTTTAAGGCAACGGTCTTGA	(Hoang et al. 2000)
Pser Down	AGTTCGGCCTGGTGGAACTCG	(Hoang et al.)

Complementing <i>algL</i> knockout		
<i>algLN</i> (NcoISDNd)	CGATCCATGGGAGGAGATAATCGCTATGAAA CGTCCCACCTGATCCGTATCG	This study
<i>algLC</i> (SmaI)	CGCTATCCCGGGCACTCAACTTCCCCCTTCGC GGCTG	This study
Confirming <i>algL</i> knockout		
<i>algL</i> upXout	GATCGAGCAGTCGAAAGCCGTCGATACC	Rehm Lab
<i>algL</i> downXout	CGTTTTCTCTGGTGTTCCTAAG	Rehm Lab

2.1.1 Long term storage of strains

Bacterial strains were grown overnight in LB (Section 2.2.1) containing appropriate antibiotics (Section 2.2.6). One millilitre of culture was transferred to a sterile 1.8 ml cryotube vial (Thermo Scientific, USA) with 70 µl of DMSO. Strains were stored at -80 °C and revived when required.

2.2 Media, growth conditions and antibiotic concentrations

The following media were used in the present study. Solid media contained agar (~95% purity, Neogen Cooperation, USA) at a final concentration of 1.5% (w/v), added before autoclaving. All media were autoclaved at 121 °C for 20 min. When required, antibiotics (Section 2.2.6) were added post-sterilization.

2.2.1 Luria-Bertani (LB) medium

LB medium was prepared by dissolving 20 g of LB powder (Acumedia, Neogen Cooperation) in 1 litre of water.

2.2.2 X-Gal medium

This was prepared by supplementing 1 L of LB medium (Section 2.2.1) with 1 mL of Isopropyl β -D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology, USA) from a stock (1 M in H₂O) and 1 mL of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-GAL) (Gold Biotechnology, USA) from a 4% (w/v) stock in DMSO.

2.2.3 Nutrient Broth

Nutrient Broth (NB) was made using Nutrient Broth Powder (Oxoid, England) as described by the manufacturer.

2.2.4 *Pseudomonas* Isolation (PI) medium

Both liquid and solid PI media were used in this study. One litre of liquid PI medium contained 20 g of peptone, 10 g of K₂SO₄, 1.4 g of MgCl₂ x 6H₂O, 25 mg of Triclosan and 20 mL of glycerol (99% purity) in 1 L of water and adjusted to pH 7.0 (Remminghorst and Rehm 2006a).

Solid PI medium (PIA), was prepared according to the manufacturer's instructions (Becton, Dickinson and Company, USA). To 1 L of water, 45 g of dry medium, 20 mL of glycerol and agar to a final concentration of 1.5% (w/v) was added prior to autoclaving. Cells were cultivated during biofilm mode on solid PIA medium.

2.2.5 Modified alginate-producing (MAP) medium

To make 1 L of MAP medium, the following chemicals were dissolved in 1 L of water (Franklin et al. 1994; Ohman and Chakrabarty 1981): 21.81 g of sodium D-gluconate, 18.70 g of monosodium glutamate, 1.17 g of NaH₂PO₄.2H₂O, 2.93 g of K₂HPO₄, 1.20 g of MgSO₄, 0.60 g of (NH₄)₂SO₄, and 0.20 g of NaCl. This solution was supplemented with 1 ml of each of the following trace mineral solutions: at 0.2 g/100 ml for each of ZnSO₄.7H₂O, CuSO₄.5H₂O, MnSO₄.H₂O and CoCl₂.6H₂O; at 0.6 g/100 ml for

FeSO₄·7H₂O, and at 0.05 g/100 ml for CaCl₂·2H₂O. The final MAP medium was filter-sterilized.

2.2.6 Supplementation of media with antibiotics

When required, media used to cultivate *E. coli* were supplemented with antibiotics at the following concentrations 100 µg/ml for ampicillin (sodium salt, Applichem, Germany), 10 µg/ml for gentamicin (sulfate salt, Applichem, Germany) and 12.5 µg for tetracycline (hydrochloride, Sigma Aldrich). For *P. aeruginosa*, antibiotics were used at 300 µg/ml for carbenicillin (disodium salt, Gold Biotechnology, USA) and gentamicin.

2.2.7 Growth conditions

All strains were grown at 37 °C. Liquid cultures (planktonic mode) were grown in Erlenmeyer flasks or universal tubes with shaking at 200 rpm in atmospheric conditions. A ratio of ≥5:1 for container to culture volume was used to support sufficient aeration. All strains cultivated on solid medium (biofilm mode) were grown at 37 °C for 24 to 72 h, as required.

2.3 DNA manipulation

General procedures were followed for DNA manipulation (Sambrook et al. 1989).

2.3.1 Isolation of plasmid DNA

Plasmid DNA was isolated from cells from 3 mL of an overnight culture, grown in LB (Section 2.2.1) with relevant antibiotics (Section 2.2.6) using the High Pure Plasmid Isolation Kit (Roche, USA) according to manufacturer's instructions.

2.3.2 Determination of DNA concentration and purity

DNA concentration and purity were assessed by fluorescence, spectrophotometry and quantitative gel electrophoresis. The Qubit™ fluorometer (Invitrogen Corporation, USA) and Quant-iT DNA BR Assay Kit (Invitrogen Corporation, USA) was used in

conjunction with the provided DNA standard to measure DNA concentration (emission/excitation wavelength of ~480/530 nm). DNA purity was assessed using a Nano-drop ND-1000 spectrophotometer (Thermo Scientific, USA). An absorbance ratio at 260/280 nm of 1.8 to 2.0 indicated high purity DNA. Quantitative gel electrophoresis was also used to estimate the concentration and purity of linear double stranded DNA fragments by comparing the relative intensity of DNA bands of serially diluted DNA samples with that of MassRuler High Range DNA ladder (Fermentas, USA).

2.3.3 Polymerase Chain Reaction

Platinum ® *Taq* and Platinum ® *Pfx* Polymerases (Invitrogen Corporation, USA) were used for analytical and preparative PCR, respectively. Reaction mixtures were prepared as outlined below (Table 2.4, page 46-47) in 0.2 ml clean sterile thin-walled PCR tubes (Axygen, USA).

Reactions were performed in a thermal-cycler 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 T_m 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

TABLE 2.4 PCR reaction mixtures

***Pfx*-DNA-polymerase reaction mixture (A)**

10X <i>Pfx</i> Amplification Buffer	20 μ l
PCRx Enhancer Solution	22.5 μ l
MgSO ₄ (50 mM)	2 μ l
DMSO	2.5 μ l
Primer 1 (10 pmoles/ μ l)	3 μ l
Primer 2 (10 pmoles/ μ l)	3 μ l
dNTPs (10 mM each)	3 μ l
Template DNA	~2 ng
Platinum® <i>Pfx</i> DNA Polymerase (2.5 U/ μ l)	1 μ l
H ₂ O	to 100 μ l

***Pfx*-DNA-polymerase reaction mixture (B)**

10X <i>Pfx</i> Amplification Buffer	20 μ l
MgSO ₄ (50 mM)	5 μ l
DMSO	5 μ l
Primer 1 (10 pmoles/ μ l)	10 μ l
Primer 2 (10 pmoles/ μ l)	10 μ l
dNTPs (10 mM each)	10 μ l
Template DNA	~2 ng
Platinum® <i>Pfx</i> DNA Polymerase (2.5 U/ μ l)	1 μ l
H ₂ O	to 100 μ l

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

Glycerol 50% (v/v)	20 μ l
DMSO	5 μ l

10X PCR Buffer (minus MgCl ₂)	10 µl
MgCl ₂ (50 mM)	10 µl
Primer 1 (10 pmoles/µl)	5 µl
Primer 2 (10 pmoles/µl)	5 µl
dNTPs (10 mM each)	10 µl
Template DNA	~2 ng
Platinum® <i>Taq</i> DNA Polymerase (5 U/µl)	1 µl
H ₂ O	to 100 µl

2.3.4 PCR dependent site-directed mutagenesis

To generate site-directed mutations, a previously described protocol was used with adaptations (Chiu et al. 2004). For each site directed mutation, two primer pairs were designed. One pair of primers were tailed (F_T and R_T), to introduce target mutations at their 5' ends, while the shorter primer pair (F_s and R_s) did not contain any mutations. For each site-directed mutation, a PCR amplification of the entire vector containing the WT *mucR* gene on pBBR1MCS-5 plasmid was performed using relevant primer pairs, generating four linear PCR products. A typical PCR reaction was composed of 2.5 µL of 10X *Pfx* Polymerase Buffer, 200 µM of each dNTP, 1 mM of MgSO₄, 100 mM of betaine, 10 pM of each primer, 103 pg of plasmid DNA (for 6.8 Kb template), 0.5 U of *Taq* DNA polymerase and 0.25 U of Platinum *Pfx* polymerase with water to a final volume of 46 µL. Reactions were run in five steps: Step 1 at 95 °C for 240 s, Step 2 at 95 °C for 15 s, Step 3 at 61 °C for 20 s, Step 4 at 68 °C for 210 s and Step 5 at 68 °C for 420 s. Steps 2-4 were repeated 25 times before Step 5, the final elongation.

To the completed PCR reaction, 4 µL of 10X buffer (Cutsmart) and 1 µL of DpnI restriction enzyme (10 U) were mixed in, and the reaction was incubated for 1 h at 37 °C. DpnI only cleaves at its recognition site if it is methylated. Since template DNA

was produced from Dam⁺ *E. coli* strain, DpnI treatment would remove the parent plasmid (which would be methylated), leaving the four PCR products intact. Figure 2.1A on page 49 schematically shows the four priming combinations and resulting PCR products (identified in parentheses) F_T/R_s (Product 1), F_s/R_T (Product 2), F_T/R_T (Product 3), and F_s/R_s (Product 4). Products 1 and 2 (resulting from primer combinations of F_T/R_s and F_s/R_T) are considered ‘productive’, because they carry the desired mutation at opposite termini, i.e. at 3’ vs 5’ ends (Figure 2.1b, page 49). In contrast, Products 3 and 4 (generated from primer pairs F_T/R_T and F_s/R_s) are ‘non-productive’ because they either have mutations at both termini (as in the case of Product 3) or no mutations at all (Product 4).

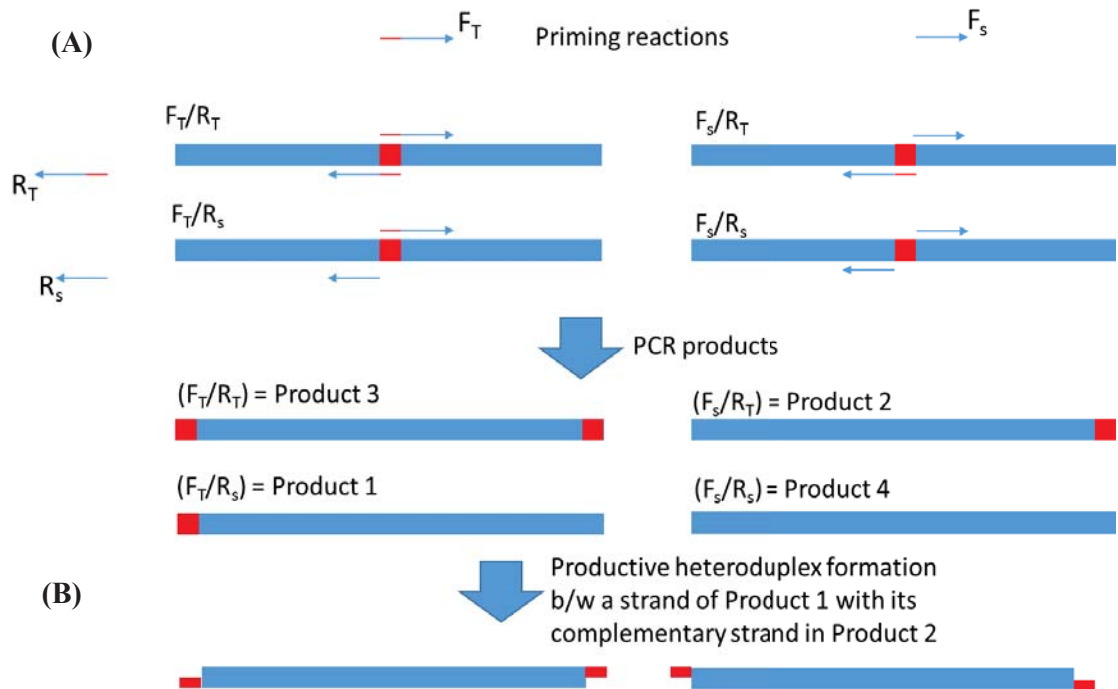


Figure 2.1 Schematic of SLIM site-directed-mutagenesis. (A) PCR was performed using template DNA (plasmid to be mutated) with two primer pairs (F_T , F_s , and R_T , R_s), yielding four PCR products; product 1 (from primers F_T/R_s), product 2 (F_s/R_T), product 3 (F_T/R_T) and product 4 (F_s/R_s). Template DNA was removed by DpnI treatment (not shown), and a re-hybridization step was introduced (B), forming productive heteroduplexes between a strand of Product 1 with its complementary strand in Product 2. These productive hybrids have complementary 5' and 3' overhangs that allow formation of stable circular DNA which was transformed and propagated in *E. coli* on selective media.

After DpnI treatment, a re-hybridization step was introduced (65 °C for 5 min and 30 °C for 15 min, for two cycles), allowing the PCR products to melt and re-hybridize, generating 16 different heteroduplexes (not shown). Among these, only re-hybridization events between one-strand of Product 1 with its complementary strand of Product 2 would generate complementary 3' and 5' overhangs at opposite termini. These 'productive' heteroduplexes form stable, non-covalently joined, DNA circles that upon transformation (Section 2.3.9) into *E. coli* could propagate on selective media.

The MHYT, GGDEF and EAL motifs of MucR were mutated to MAYT, GGAAL and AAL, respectively, using relevant primers, listed in Table 2.3, page 39-41.

2.3.5 Hydrolysis of DNA by restriction endonucleases

Plasmid DNA (Section 2.3.1) and PCR products (Sections 2.3.3 and 2.3.4) were hydrolysed by restriction endonucleases (REs) for cloning and analytical purposes. Restriction endonucleases were used according to the manufacturer's instructions (New England Biolabs, USA). Preparative and analytical digests were performed in clean sterile microtubes containing 1X concentration of the relevant buffer.

In preparative digests 100 µg of DNA was digested with 100 U of enzyme in 50 µL reactions while in analytical digests 10 U of enzyme was used to hydrolyse 1 µg of plasmid DNA in 20 µL reactions. Unless otherwise stated, all digestions were performed at 37 °C for 2-4 h and stopped by adding 0.2 vol of 6× stop-mix containing Tris-HCl (pH 8.0) at 60 mM, EDTA at 60 mM, glycerol at 60% (v/v), and Orange G at 0.2% (w/v) and Xylene Cyanol FF at 0.05% (w/v) (Sambrook et al. 1989).

2.3.6 Agarose gel electrophoresis

Agarose gel electrophoresis (AGE) was used to assess DNA concentration and purity (Section 2.3.2), and separate DNA fragments after PCR (Section 2.3.3) and restriction

endonuclease hydrolysis (Section 2.3.5). When needed, DNA could be recovered from gels (Section 2.3.7). Agarose gels of 1% and 2% (w/v) were used to resolve double stranded DNA fragments above and below 500 bp, respectively. Agarose (HYAgarose, HydraGENE Co. Ltd. USA) was added to TBE electrophoresis buffer (50 mM Tris-HCl, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) and melted in a microwave oven, poured into a gel chamber, and a well comb was inserted. Upon solidification (after 1 h) the gel comb was removed. The chamber together with gel was placed into an electrophoresis apparatus, submerging the gel in TBE buffer. To each volume of DNA sample, 0.2 vol. of a 6× stop-mix was added (see section above) prior to loading.

A DNA molecular size standard was included in a separate well. Molecular size markers used in this study were Lambda phage DNA hydrolysed with PstI (Sambrook et al. 1989), MassRuler High Range DNA ladder (Fermentas, USA), and 100 kb + Ladder (Invitrogen, USA). Gels were run 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 seen using an ultra violet (UV) transilluminator and pictures generated (Bio-Rad, Gel Doc, USA).

2.3.7 Isolation of linear dsDNA

To isolate linear dsDNA from polymerase chain reactions (Section 2.3.3) or restriction endonuclease digestions (Section 2.3.5), DNA was subject to AGE (Section 2.3.6) in gels containing 1X concentration of SYBR™ Safe DNA Gel Stain (Invitrogen, USA). Under UV light, the target DNA band was excised using a sterile scalpel blade. Target DNA was purified from the gel using the Zymo Gel DNA Recovery Kit (Zymo Research, USA) according to manufacturer's instructions.

2.3.8 DNA ligation

Insert DNA obtained from PCR (Section 2.3.3) or RE digestions (Section 2.3.5) were recovered from AGE (Section 2.3.7). Ligation reactions of insert with linearized 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. Reactions were incubated at 16 °C overnight.

2.3.9 DNA sequencing

All DNA sequencing of recombinant plasmids was provided by the Massey Genome Service utilizing the dGTP BigDye Terminator v 3.0 chemistry in 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 purified plasmid DNA (Section 2.3.1) was suspended in 20 µl of molecular grade water, containing 4 pMol of the relevant primer, 1 µL of DMSO, and sent for sequencing in 0.2 ml clean sterile thin walled PCR tubes (Axygen, USA).

2.3.10 Transformation of *E. coli*

The recipient *E. coli* strain was cultivated in 50 ml of LB (Section 2.2.1), with appropriate antibiotics (Section 2.2.6), to an optical density at 600 nm (O.D 600 nm) of 0.3. After keeping on ice for 10 min, cells were harvested by centrifugation (4,000 g at 4 °C for 20 min). The cell pellet was suspended in 18 ml of RF1 solution and kept on ice for 1 h. Cells were harvested again and suspended in 4 mL of RF2 solution. Subsequently, 200 µL aliquots were transferred into 1.7 mL microtubes, frozen in liquid nitrogen and stored at -80 °C. The above RF1 and RF2 solutions were sterilized by filtration (0.22 µm). RF1 solution contained RbCl at 100 mM, MnCl₂ at 50 mM,

Potassium acetate at 30 mM and $\text{CaCl}_2 \times 6 \text{H}_2\text{O}$ at 10 mM dissolved in water and adjusted to pH 5.8 with acetic acid. RF2 solution was composed of RbCl at 10 mM, MOPS at 10 mM, $\text{CaCl}_2 \times 6 \text{H}_2\text{O}$ at 75 mM and glycerol at 15% (v/v), solubilized in water and 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 and incubated on ice for 1 h. After subjecting cells to heat shock (42 °C for 90 s), tubes were incubated on ice for 5 min. Subsequently, 800 µL 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), suspended in 200 µl of LB medium and plated on LB agar containing relevant antibiotics (Section 2.2.6) to select for colonies containing the plasmid of interest.

2.3.11 Transconjugation of *P. aeruginosa*

Transfer of conjugation-competent-plasmids into *P. aeruginosa* strains was performed as outlined previously (Friedrich et al. 1981). The donor strains *E. coli* S17-1 was used to transfer plasmids harbouring the Mob (oriT) region. Firstly, the plasmid of interest was transformed into the *E. coli* donor strain (Section 2.3.9). Donor *E. coli* and recipient *P. aeruginosa* strains were grown separately in 20 ml of LB medium (Section 2.2.1) overnight, with appropriate antibiotics (Section 2.2.6). Cells were harvested by centrifugation (4,000 g at 4°C for 20 min) and re-suspended in 0.9% (w/v) sterile saline. Cells were washed thrice with sterile saline to remove extracellular polymeric substances that may interfere with conjugation. Washed cells were re-suspended in 1 ml of sterile saline, 200 µl of the donor and recipient suspensions were combined in sterile 1.7 ml micro-centrifuge tubes. After mixing by inversion, cells were harvested via centrifugation (15,000 g for 2 min), re-suspended in 200 µl of sterile saline and dropped carefully using a 1 ml pipette onto an NB agar plate (Section 2.2.3). After five minutes,

plates were gently transferred to 37 °C and incubated for 24 h. After incubation, a sterile spatula was used to scrape biomass from plates. Cells were then suspended in 1 ml of sterile saline and after a 1,000-fold dilution (no dilution required for pEX100T derived plasmids), 100 µl of cell suspensions were plated on PIA medium (Section 2.2.4) supplemented with relevant antibiotics (Section 2.2.6) and carbon source to select for successful tranconjugants.

2.4 Generation of isogenic marker free mutants and complemented strains

A commonly used homologous recombination gene replacement strategy had been used to knock out target genes involved in alginate biosynthesis (*algL*) and regulation (*mucR*). Briefly, to disrupt each gene, plasmid borne ‘knockout cassettes’ were used. For each gene, its respective ‘knockout cassette’ consisted of a gentamicin resistance marker flanked by two regions of the gene; each region being ~300-500 bp long. These fragments were obtained by PCR and ligated on either side of the resistance marker, into the delivery vector, pEX100T. The marker itself is immediately flanked by two flip recombinase recognition sites, allowing the marker to be removed after gene disruption. The delivery vector pEX100T (Hoang et al. 1998), harbours an *oriT* (origin of transfer) to facilitate conjugation and a *sacB* gene that confers lethality on sucrose containing media. Final knockout vectors, pEX100T Δ *mucR* Ω Gm^R and pEX100T Δ *algL* Ω Gm^R were introduced into donor *E. coli* strain SM10 (Section 2.3.9) for conjugation into PDO300 (Section 2.3.10).

Recipients were grown on PIA medium containing gentamicin (to select for integration of the marker), and sucrose (to select for a double cross over event). Disruption of the target gene (by a double cross over event, replacing part of the target gene with the gentamicin resistance marker) was verified by growth on gentamicin containing medium and colony PCR using primers flanking the target gene of interest (Table 2.3, page 39). The marker was subsequently removed by introducing a flip recombinase containing plasmid, pFLP2 by conjugation (Hoang et al. 1998). Successful recipients were selected for on PIA medium containing carbenicillin. Excision of the marker was confirmed by colony PCR and sensitivity to gentamicin. Since the pFLP2 plasmid also harbours a *sacB* gene, it could be removed by counter selection on sucrose containing media. Final strains, PDO300 Δ *mucR* and PDO300 Δ *algL*, were confirmed by colony

PCR, growth on sucrose containing medium and susceptibility to carbenicillin and gentamicin. The knockout plasmids pEX100T Δ *mucR* and pEX100T Δ *algL* were constructed by Iain Hay and Uwe Remminghorst, respectively. The mutants were PDO300 Δ *mucR* and PDO300 Δ *algL* generated by Iain Hay and Ali Goudarztalejerdi, respectively (Hay et al. 2009b; Wang et al. 2016a).

For complementation, genes of interest were expressed *in trans* on various plasmids. For complementation of the *algL* mutant, the variants of the *algL* gene (encoding either catalytically active or inactive variants of AlgL) were expressed from arabinose inducible promoters on a medium copy number plasmid pHERD20T, that carried a carbenicillin resistance marker. For the *mucR* investigation, variants of *mucR* were expressed from a moderate constitutive promoter on a medium copy number plasmid pBBR1MCS-5, carrying a gentamicin resistance marker. Variants of *algL* and *mucR* were generated by a combination of PCR (Section 2.3.3), site-directed mutagenesis (Section 2.3.4) and gene synthesis.

2.5 Alginate analysis

Alginate yield, molecular weight and composition were assessed by a uronic acid assay, SEC-MALLS and $^1\text{H-NMR}$, respectively. In some previous studies alginate production was assessed by the gain/loss of mucoidity (Boyd et al. 1993), a colorimetric carbozole assay (Albrecht and Schiller 2005; Jain and Ohman 2005; Monday and Schiller 1996) or alginate lyase assay – based on increased absorbance at 230 nm associated with beta-elimination (Bakkevig et al. 2005). In the present work, a previously described uronic acid assay was used because it displayed greater sensitivity and specificity than the carbozole assay, the latter of which is susceptible to interference by neutral sugars (May and Chakrabarty 1994). Moreover, the loss/gain of mucoid phenotype can be subjective and cannot reveal relative changes in alginate yield. Lastly the alginate-lyase assay is susceptible to background absorbance of contaminants including other carbohydrates, peptides, phenols or aromatic compounds. Thus, there is reason to believe that the uronic acid assay here is more accurate and reliable than the methods used by previous groups.

2.5.1 Preparation of samples from solid medium

Alginate produced by strains grown on solid medium (biofilm growth mode) was harvested (Remminghorst and Rehm 2006a), with modifications, which helped standardise biomass of the initial inoculum and eliminate effects of medium quantity and supernatant carryover. A loop full of frozen culture stock (Section 2.1.1) was used to inoculate 5 ml of LB medium containing antibiotics. After 14 h incubation, optical density was measured (at 600 nm) and sufficient volume of this culture was added to 20 ml of fresh LB medium (Section 2.2.1) supplemented with appropriate antibiotics (Section 2.2.6) to obtain an initial O.D. of 0.05. After 14 h incubation at 37 °C, cells were harvested by centrifugation (8,000 g for 30 min), and washed twice with 20 ml

sterile saline. This washing step was included since previous studies have shown that carry-over of supernatant components substantially affects alginate characteristics (Trujillo et al. 2003b). Cells were re-suspended to an optical density of 6.0 and 1 ml of cells was suspended in 200 μ L of sterile saline and plated onto PIA medium (Section 2.2.4) containing relevant antibiotics (Section 2.2.6). For optical densities over 0.3, ten-fold serial dilutions were used.

After 72 h incubation, biomass from plates was scrapped off, suspended in 150 ml of sterile saline and left on a horizontal shaker for 60 min. Cells were removed 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 (96% v/v) (Sigma Aldrich, USA). The cell pellet and crude alginate precipitates stored at -80 °C and then freeze-dried in a floor-model freeze-dryer (Dura-Dry MP Model # FD2085C0000, FTS Systems, USA). Alginate samples were solubilised in buffer (0.05 M Tris-HCl, 10 mM MgCl₂) to a final concentration of 0.5% (w/v). To remove co-precipitated nucleic acids and proteins, samples were incubated with DNase I (15 μ g/ml) and RNase A (15 μ g/ml) for 6 h at 37 °C (with shaking at 200 rpm) followed by a further 18 h incubation with Pronase E (20 μ g/ml). Alginates were then dialysed (Dialysis Tubing, high retention seamless cellulose tubing, 40 mm width, MW cut off: 12,400 kDa) against 5 litres of 0.9% w/v NaCl in water for 24 h at 4°C. Dialysed alginate samples were precipitated with ice-cold isopropanol, freeze-dried and weighed. These samples were dissolved in 200 μ L of water at 250 μ g/ml and uronic acid content was measured (Section 2.5.3).

Alternatively, alginate samples for molecular mass determination (Section 2.5.4) and compositional analysis (Section 2.5.5) were dialysed against 5 L of water prior to precipitation, as above.

2.5.2 Preparation of samples from liquid medium

Alginate produced by strains grown in liquid medium (planktonic growth mode) was measured as described previously (Rehman et al. 2013), with modifications. Strains were grown for 14 h in LB medium containing antibiotics. From this pre-culture a sufficient volume was added to 20 mL of MAP medium (Section 2.2.5) supplemented with appropriate antibiotics (Section 2.2.6) to produce a culture with an initial O.D 600 nm of 0.05. Cultures were incubated for 24 h and cells were harvested by centrifugation (15,000 x *g* for 1 h). The uronic acid (UA) content of filtered and unfiltered supernatants was determined (Section 2.5.3). To filter supernatants a 10 kDa cut off (Vivaspin® 500 Centrifugal Concentrator, GE Healthcare Life Sciences, USA) filter was used, according to manufacturer's instructions. UA content of unfiltered supernatants represented the total alginate yield, inclusive of high and low molecular weight (MW) alginate while the UA content of filtered supernatants represented the low MW alginate that is poorly polymerised and/or degraded.

2.5.3 Uronic acid analysis

Uronic acid content of samples was measured using a colorimetric assay (Blumenkrantz and Asboe-Hansen 1973) using alginic acid sodium salt from brown seaweed as a standard (Sigma-Aldrich, USA). Samples (200 µL) prepared according to Sections 2.5.1 and 2.5.2 were added to 1.2 ml of tetraborate solution (0.0125 M tetraborate in concentrated sulphuric acid), vortexed immediately and placed on ice for 10 min, at 100 °C for 5 min, and then put back on ice for another 5 min. To this mixture, 20 µl of *m*-hydroxybiphenyl reagent (Sigma Aldrich, USA) (0.15% w/v *m*-hydroxybiphenyl in 0.125 M NaOH) was added and samples were vortexed for 1 min. For each sample and standard, a negative control was included by substituting the *m*-hydroxybiphenyl

reagent with 20 μ l of 0.125 M NaOH. Absorbances were measured at 520 nm for each sample and standard, using the negative controls as blanks.

2.5.4 Molecular mass determination

Alginate molecular masses were determined as previously described (Moradali et al. 2015). For each strain, alginate from six PIA plates was harvested (Section 2.5.1), pooled and subjected to Size Exclusion Chromatography-multi-Angle Laser Light Scattering (SEC-MALLS) analysis. A Waters 2690 Alliance separations module and a DAWN-EOS multi-angle laser light scattering detector with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA, USA); Waters 2410 refractive index monitor) were used. Purified samples (Section 2.5.1) were dissolved in 0.1 M NaNO₃ (2 mg/mL) and allowed to hydrate fully by incubating them overnight at room temperature. Immediately prior to analysis, samples were pre-heated at 80 °C for 5 min, injected (100 μ L) and eluted with 0.1 M NaNO₃ (0.7 mL/min, 60 °C) from two columns (TSK-Gel G5000PWXL and G4000PWXL, 300 \times 7.8 mm, Tosoh Corp.) connected in series. ASTRA software (version 6.1.2.84, Wyatt Technology Corp.) and dn/dc of 0.150 mL/g was used for determining weight-average molecular weights (M_w) and number-average molecular weights (M_n) and polydispersity index via the fraction M_w/M_n . In the case of a uniform polymer, polydispersity index equals 1.0.

2.5.5 Compositional analysis

Alginate O-acetylation degree and M/G ratio and frequency of MM, MG/GM and GG diads was determined by ¹H-nuclear magnetic resonance (¹H-NMR) spectroscopy (Moradali et al. 2015). For each strain, alginate from six PIA plates was harvested (Section 2.5.1), pooled and subjected to ¹H-NMR analysis. Samples were prepared and spectra were recorded at 90 °C with a JEOL 270 ¹H-NMR spectrometer (6.34 T) operating at 270 MHz for proton. Chemical shifts were expressed in parts per million

downfield from the signal for 3-(trimethylsilyl) propanesulfonate (Sigma Aldrich, USA). Compositional differences of de-acetylated and acetylated alginate samples were determined by integration of $^1\text{H-NMR}$ signals. Acetyl-groups were removed by alkaline treatment where 30 ml of 1% alginate samples in saline solution were treated with 12 ml of 1 M NaOH at 65 °C for 30 min followed by neutralization with 12 ml of 1 M HCl. These samples were dialysed against 5 L of distilled water for 48 h and then freeze-dried. De-acetylated and acetylated samples were compared by $^1\text{H-NMR}$ to determine degree of O-acetylation.

2.5.6 Alginate lyase assay

Detection of alginate lyase activity in cultures grown on solid media was performed as described by (Sawant et al. 2015), with modifications. Strains were grown on PIA medium for 72 h. Biomass was suspended in saline and cells were pelleted by centrifugation. Supernatants were filter-sterilized and 100 μl of cell-free re-suspensions were dropped onto alginate plates [LB medium supplemented with 1% w/v agarose and 0.1% w/v sodium alginate (Sigma Aldrich, USA)] for 24 h at 37 °C. Plates were flooded overnight with 10% w/v cetylpyridinium chloride. The appearance of clearings indicated alginate degradation. Commercial alginate lyase (Sigma Aldrich, USA) was used as positive control (5 μL of 10 mg/ml stock).

2.6 Protein analysis

2.6.1 Cultivation of strains for protein analysis

Strains were cultivated in a similar manner as described in Section 2.5.1 for alginate analysis. A loopful of frozen culture stock (Section 2.1.1) was used to inoculate 5 ml of LB medium containing antibiotics. After 14 h incubation, optical density was measured (at 600 nm) and sufficient volume of this culture was added to 20 ml of fresh LB medium (Section 2.2.1) supplemented with appropriate antibiotics (Section 2.2.6) to obtain an initial O.D of 0.05. After 14 h incubation, cells from this pre-culture were harvested by centrifugation (8,000 g for 30 min), and washed twice with 20 ml sterile saline. Afterwards, cells harvested from the pre-culture were re-suspended to an optical density of 6.0. For each treatment, 1 ml of each standardized cell-preparation was spread onto a PIA plate (Section 2.2.4) containing relevant antibiotics (Section 2.2.6), using a sterilized glass spreader. Strains were grown on solid medium for 72 h, biomass from plates was scrapped off, suspended in 150 ml of sterile saline and left on a horizontal shaker for 60 min. Cells were harvested by centrifugation (9,000 g at 4 °C for 45 min).

2.6.2 Protein extraction

To obtain cell lysates, *P. aeruginosa* cell samples (Section 2.6.1) were treated on ice for 1 h with Buffer A (10 mM HEPES, 150 mM of NaCl, pH 7.4) supplemented with $\times 1$ conc. of Roche EDTA free protease inhibitor and 1.5 $\mu\text{g/ml}$ of lysozyme. Cells were lysed by sonication (on ice, 12 cycles—15 s of sonication and 15 s cool down). Unlysed cells and debris were removed by centrifugation (15,000 g for 30 min at 4 °C). To sediment the whole envelope fraction, the clear cell lysate was ultra-centrifuged (100,000 g for 90 min at 4 °C) and the pellet containing the membrane fraction was suspended in 300 μl of Buffer A.

When required, the inner membrane fraction was further enriched by adding 0.7% w/v N-lauroyl-sarcosine (Sigma Aldrich, USA) to Buffer A. Samples were mixed by gentle agitation at 25 °C for 1 h to solubilize the inner membrane fraction. Subsequent ultracentrifugation (100,000 *g* for 90 min at 4 °C) was performed to sediment the outer membrane fraction, which was discarded, leaving the inner membrane fraction in the supernatant.

Protein samples were then flash frozen in liquid nitrogen in 50 µL aliquots and stored at -80 °C for later analysis.

2.6.3 Protein quantification

Protein concentrations were determined using a commercially available Bradford Assay according to manufacturer's instructions (Bio-Rad, USA). Bovine serum albumin at concentrations of 0.80 to 0.05 mg/ml (in the same buffer used to suspend protein samples) was used to generate a standard curve. Serial two-fold dilutions of samples (up to ×32) were made in 96 well plates. Ten microlitres of standards and samples was individually mixed with 200 µl of Bradford reagent (Bio-Rad, USA) in separate wells. Plates were incubated at room temperature for 5 min and the absorbance at 595 nm was measured using a plate reader (ELx808iu Ultramicroplate Reader, Bio-Tek instruments, USA).

2.6.4 Crosslinking

A crosslinking reagent was used to 'capture' protein-protein interactions and assess their oligomeric states. The cross-linker used in this study, disuccinimidyl glutarate (DSG) from Thermo Scientific Pierce, USA, is a water-soluble, homo-bifunctional *N*-hydroxysuccinimide ester (NHS-ester) based crosslinker. This moiety reacts with primary amines on the *N*-termini of peptides and the ε-amine of lysine residues, forming stable covalently linked amide bonds, while releasing NHS groups. DSG harbours two

such moieties which are positioned separately at opposite ends of a linker spacer arm with a length of 7.7 Å.

DSG was used in crosslinking reactions to investigate protein-protein interactions and/or protein oligomeric states. In general, strains were grown for 72 h on PIA media (Section 2.2.1) containing relevant antibiotics (Section 2.2.6). Biomass from three plates were harvested and washed six times in buffer (10 mM HEPES, 150 mM NaCl, pH 7.7). To assess effect of nitrate on MucR oligomeric state, cells grown in the presence of nitrate were washed with the same buffer supplemented with 50 mM of KNO₃. Cells were incubated for 30 min at 37 °C in 5 ml of crosslinking buffer (10 mM HEPES, 150 mM NaCl, pH 7.4, 20% v/v of DMSO, 5 mM of DSG). For each treatment, a negative control (i.e. blank) was included; these cells were suspended in crosslinking buffer minus DSG. Reactions were stopped by addition of Tris-HCl to a final concentration of 20 mM at pH 7.4. Cells were harvested by centrifugation and protein samples were prepared and analysed as described in Sections 2.6.1 to 2.6.3.

2.6.5 Hexahistidine Pull Down

Another approach to studying protein-interactions is by expressing hexahistidine-tagged ‘bait’ proteins in cells followed by purification of the tagged-bait protein via nickel ion affinity chromatography (NIAC), and searching for candidate interaction-partners in eluted samples through SDS-PAGE and immunoblot analysis.

For such assays, strains were cultivated for 72 h on PIA medium supplemented with relevant antibiotics. Biomass was harvested and cells were washed with saline. The envelope fractions of cell lysates were obtained as outlined in Section 2.6.1 using Buffer B (in place of Buffer A), supplemented with 0.1 mg/ml of lysozyme and 0.1 mg/ml of DNase A. (Buffer B is composed of 100 mM phosphate buffer, 150 mM NaCl, 5 mM imidazole, 5 mM EDTA, 1X Roche EDTA-free protease inhibitor, 10% v/v glycerol).

Unlysed cells and cell debris were removed by centrifugation at 15,000 *g* for 45 min at 4 °C. From cleared cell lysates, envelope fractions were prepared by ultracentrifugation, solubilized in Buffer C (Buffer B supplemented with 0.2% v/v of Triton X-100T) for 1 h on ice, and subject to NIAC purification using a commercially available resin (cOmpleteTM His-Tag Purification Resin, Roche, USA), in conjunction with a Zymo-Spin P1 Column and Collection Tube (Zymo Research, USA). Unbound proteins were removed by centrifugation (10,000 *g* for 10 s) and impurities were removed by four washes with Buffer C. To elute His-tagged proteins, resin was incubated with Buffer D (Buffer B containing 400 mM imidazole) on ice for 10 min then centrifuged.

The purification resin used is fully compatible with metal chelators (e.g. EDTA) and reducing agents (e.g. β -mercaptoethanol), and resilient to extreme pH (from 2-14) and chaotropic agents (e.g. 8 M urea).

2.6.6 SDS-PAGE

Extracted protein samples (Section 2.6.2) were mixed with 0.2 vol of 6X SDS-PAGE loading buffer and heated to 95 °C for 10 min. A milliliter of 6X SDS-PAGE loading buffer contained 500 μ l of glycerol, 120 mg of SDS, 93 mg of DTT, 0.2 mg of Bromophenol blue and 500 μ l of 4X Stacking Buffer (Laemmli 1970) After sample preparation, 10-20 μ g of each protein sample was run on SDS-PAGE.

A discontinuous buffering system was used for SDS-PAGE (Laemmli 1970). For this method, stock solutions of 1X electrophoresis buffer (3 g of Tris, 1 g of SDS and 14.4 g of glycine in water, adjusted to pH 8.5, in 1 L of water) and 4X stacking gel and 4X separating gel were prepared. One liter of 4X stacking gel buffer contained 81.7 g of Tris and 4 g of SDS in water, adjusted to pH 8.9. One liter of 4X separating gel buffer contained 60.6 g of Tris and 4 g of SDS in water, adjusted to pH 6.8. Utilizing these buffers, two standard 1.5 mm SDS-PAGE gels composed of 8.0 and 3.9% (w/v)

acrylamide separating and stacking gels were prepared as follows: For a 20 ml preparation of an 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 Na₂SO₃ 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, the mixture was stirred for 10 s before it 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 Na₂SO₃ was added to degas the mixture. Then, 2.5 µl of 40% (w/v) APS and 5 µl of TEMED were added, the mixture was stirred for 10 s and then poured onto the separating gels and a 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 and loaded into separate wells. For each gel, a protein molecular weight standard (3-4 µL of either GangNam-STAIN Prestained Protein Ladder (intRON Biotechnology, USA) or Novex™ Sharp Pre-Stained Protein Standard (Invitrogen, USA) was included. Pairs of 1.5 mm SDS-PAGE gels were run at 150 V for 60-90 min.

2.6.7 Immunoblot

To identify Alg proteins (Alg44, K, X, E and L), samples were run on SDS-PAGE (Section 2.6.6), transferred to nitrocellulose membrane, probed with relevant primary

and secondary antibodies and visualized by chemical-luminescence through exposure and development of auto-radiographic film.

Proteins from SDS-PAGE were transferred to nitrocellulose membrane using a semi-dry transfer system (iBlot® Gel Transfer Stacks Nitrocellulose and iBlot® Gel Transfer System, Invitrogen Corporation, USA). The iBlot® Anode Stack was placed on the apparatus according to the company's instructions. This Anode Stack has a layer of nitrocellulose membrane resting on a buffer-infused mesh coated on the underside by a thin sheet of copper – the anode. Onto the nitrocellulose membrane of the anode stack the SDS-PAGE gel was placed followed by a layer of moistened iBlot® Filter Paper (with molecular grade water). This was followed by the iBlot® Cathode Stack (a buffer-infused mesh coated on the upper-side by a thin sheet of copper – the cathode) and the iBlot® Sponge (to apply even pressure while completing the circuit). The supplied 'roller' was used between application of each layer to remove 'air-bubbles'. The lid was then securely fastened, and transfer programme No. 3 was initiated.

After transfer, the nitrocellulose membrane was washed thrice in a clean square-plastic-petri dish 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 at room temperature for 1 h in TBST + 5% (w/v) BSA. The membrane was then washed three times and incubated overnight at 4 °C with relevant rabbit anti-Alg antibody in TBST + 1% (w/v) BSA. For Anti-Alg44, K, E and L antibodies, antibody serum to buffer volume ratios of 1:10,000 (v/v) were used, while for the Anti-AlgX antibody, 1:7,000 was used. After incubation, the membrane was washed again (x3 for 5 min) with TBST and incubated in the dark with goat anti-rabbit antibody conjugated to horse radish peroxidase (Abcam, England) at a 1:10,000 (v/v) ratio in TBST + 1% (w/v) BSA. After washing three times again, membranes were incubated for 5 min in the dark with 3 ml

of substrate and 3 ml of enhancer solutions (Super Signal West Pico Stable Peroxide Solution and Luminol/Enhancer Solution, Thermo Scientific, USA). All incubations/washes were done with gentle agitation. To visualize bands, membranes were exposed to BioMax XAR film (Kodak, USA) and images were developed (Kodak X-Omat-100, USA).

For detection of his-tagged proteins a commercially available Anti-His antibody conjugated to horse radish peroxidase (HisProbe-HRP Kit, Thermo Scientific, USA) was used as described by the company. Nitrocellulose membranes were blocked overnight in TBST + BSA at 1% (w/v). Membranes were washed twice in 15 ml of TBST and incubated with the antibody at 1:5,000 (v/v) in TBST for 1 h at room temperature in the dark. Following another four washes with TBST, the membrane was incubated with the substrate and images were obtained as outlined above.

2.7 Swarming Motility

Swarming motility was assessed as described previously (Tremblay et al. 2007). Five microliters of an overnight culture were used to inoculate the centre of each swarming plate. These plates consisted of modified M9 medium: 20 mM of NH_4Cl , 12 mM of Na_2HPO_4 , 22 mM of KH_2PO_4 , 8.6 mM NaCl , 1 mM MgSO_4 , 1 mM of CaCl_2 , 11 mM of dextrose and 0.5% (w/v) of casamino acids (Difco) - solidified with agar (0.5% w/v). Autoclaved medium was poured in petri dishes and allowed to solidify under laminar flow for 1 h. Inoculated plates were incubated at 30 °C for 16 h. In the context of assessing the effect of nitrate on motility, plates were supplemented with and without 1% w/v of KNO_3 . Motilities were recorded by photography. A negative result was demonstrated by no swarming (no expansion of the colony, i.e. confinement of cells at the initial site of inoculation). A positive result was scored if colonies expanded i.e.

forming a typical swarming appearance as defined by formation of dendritic fractal-like patterns by migrating swarms moving away from initial location.

2.8 Biofilm analysis

Biofilm growth was analysed by two methods, (1) a 96 well plate assay (Section 2.8.1) and (2) via flow chamber growth (Section 2.8.2). In the latter case, biofilms grown on glass plates were analysed by confocal laser scanning microscopy (Section 2.8.3) and IMARIS software (Section 2.8.3).

2.8.1 96 well plate assay

A 96 well plate assay was used to study solid surface attachment, biomass growth and biofilm dispersal (Merritt et al. 2005). For these assays, strains were grown in 10 mL of PI medium containing carbenicillin and arabinose in 125 mL flasks at 200 rpm (shake model) 37 °C for 24 h. To assess initial attachment, cultures were standardized to an O.D 600 nm of 2.0 using respective culture supernatants. 100 µL of standardized cultures were added into eight wells of a clean sterile 96 well plate and incubated with lid on for 2 h at 37 °C. Unbound cells were washed off by gently rinsing the plate twice under tap water, after which plates were inverted and left to dry at ambient temperature.

Subsequently 150 µL of crystal violet solution (crystal violet at 1% w/v in water) was added into each well. After 10 mins unbound stain was removed by gently rinsing the plate twice with tap water and then plates were air dried. Subsequently 200 µL of DMSO was added to each well, and after 10 mins the absorbance at 595 nm was measured.

To assess biofilm growth, the above 24 h PI cultures were diluted with fresh PI medium containing carbenicillin and arabinose to optical densities of 0.05. 100 µL of these suspensions were used to inoculate individual wells of a 96 well plate (4 wells for each

treatment). To reduce the effect of medium evaporation on cell growth, the remaining empty wells were filled with 200 μ L of sterile water. After incubating plates for 72 h at 37 °C with the lid on, unbound cells were washed off and bound cells/biomasses were stained as described above.

To investigate the dispersal efficiency of strains, 24 h PI cultures were diluted with fresh PI medium containing carbenicillin and arabinose to an O.D of 0.200. 100 μ L of these suspensions were used to inoculate individual wells and incubated for 24 h. For each treatment, four wells were used. To reduce the effect of medium evaporation on cell growth, the remaining wells were filled with 200 μ L of sterile water. After 24 h incubation at 37 °C, unbound cells were removed by washing twice with water. To examine dispersal efficiency, wells were incubated with 200 μ L of sterile saline for 1 h. Subsequently, 10 μ L of supernatant was mixed with 990 μ L of sterile saline in clean sterile 1.7 ml Eppendorf tubes. Of this suspension, 100 μ L was spread onto a PIA plate containing relevant antibiotics. Agar plates were cultivated overnight and colony forming units determined.

2.8.2 Flow chamber set-up

To study *P. aeruginosa* biofilm formation, strains were grown on a glass slide in continuous-culture flow cell channels (with dimensions of 4 mm \times 40 mm \times 1.5 mm) at 37 °C (Hay et al. 2009a). This concept is illustrated schematically on page 71 in Figure 2.2 (Nielsen et al. 2011).

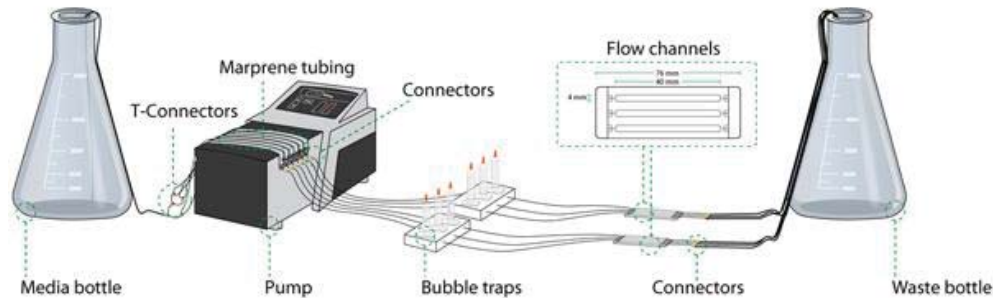


Figure 2.2 Schematic of flow cell chamber experimental set up. The growth chamber (flow channel) was assembled and connected to the rest of the assembly. The media bottle, pump, bubble trap, flow cells and waste bottle were connected by various tubing. Image reused with permission from JoVE (Nielsen et al. 2011).

Flow cells were assembled by gluing the plastic-flow-chambers with a glass-cover-slip using water-tight silicon-glue (Selleys All Clear Multipurpose Co-Polymer Sealant). After allowing a couple of days for the glue to harden, chambers were submerged in 70% v/v ethanol to maintain sterility. Autoclavable parts of the system (media and tubing) were prepared and sterilized by autoclaving. To assemble the flow-cell-chamber system, the media input container was connected by tubing, and divided into eight Marprene tubes using T-connectors, each clipped onto a laminar flow-pump to control flow rate. These tubing were connected to separate bubble traps and which were connected by further tubing to individual flow chamber channels units. Output tubing from channels were connected to distribute waste-medium into a discharge container, which was also kept 'above' the assembly to avoid accumulation of bubbles in growth chambers. Overall, growth chambers were kept 'below' the rest of the assembly to minimize bubbles.

The assembled system was sterilized by flushing twice then incubating overnight with hypochlorite (0.5% v/v) solution. Prior to inoculating the system, hypochlorite was washed out with sterile water, then flushed and infused with the growth medium.

Cultures used for inoculating growth chambers were grown overnight to early-stationary phase in LB medium (Section 2.2.1) supplemented with relevant antibiotics (Section 2.2.6). 0.5 ml of cultures were injected into the input tubing immediately upstream of the growth chamber channel. To do this, immediately upstream of the injection site, a bull-dog clip was used to clamp the tubing shut to prevent culture from contaminating upstream media. Injection sites were sterilized with 70% (v/v) ethanol, and cultures were slowly injected into tubing towards the growth chambers using sterile 1 mL syringe and needle. After delivering the injection, the site was promptly sealed with water-tight silicon glue.

Injected cells were allowed to attach to the glass surface for 4 h at 25 °C without flow. After allowing cells to bind to the surface, a flow rate of 0.3 ml/min (Reynolds number of 5) was maintained for 20 h, using PI medium.

2.8.3 Confocal laser scanning microscopy and IMARIS analysis

Cells grown in flow cell chambers (Section 2.8.2) were then stained using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) to visualize cells that were either living (green) or dead (red), using confocal laser scanning microscopy (CLSM) (Leica SP5 DM6000B). This dye mixture contains two fluorescent dyes SYTO 9 (green) and propidium iodide (red) which bind to DNA. The former stain is membrane permeable (stains living cells) while the other is non-permeable (stains dead/damaged cells). With excitation/emission maxima of 480/500 for SYTO 9 and 490/635 for propidium iodide, live cells would appear green and dead

cells red when viewed by CLSM. Biofilm characteristics were analysed by IMARIS software as described (Ghafoor et al. 2011), obtaining the average biofilm height and compactness. A ratio of dead to live cells was calculated by dividing the total red (dead) with the total green (live) fluorescence (Ghafoor et al. 2011).

2.9 LacZ reporters

Various promoter- and c-di-GMP-sensitive *lacZ* reporters were used in this study as a convenient, non-disruptive way of measuring gene expression and intracellular c-di-GMP levels.

2.9.1 Promoter reporter

Promoter activities of *algD*, *pelA*, and *pslA* were determined by previously constructed promoter-specific *lacZ* fusion reporter plasmids: miniCTX*P_{algD}lacZ*, pTZ110:*pelA*, and pTZ110:*pslA* (Ghafoor et al. 2011; Hay et al. 2012; Overhage et al. 2005). Promoter activities were inferred from beta-galactosidase assay (Section 2.9.3).

2.9.2 c-di-GMP reporter

To assess global intracellular concentrations of c-di-GMP, a c-di-GMP-sensitive *lacZ* reporter plasmid was made as described earlier (Baraquet et al. 2012). This plasmid, mini-CTX-*lacZ-pelAwt*, was introduced into relevant *P. aeruginosa* strain and its integration into the genome was confirmed by colony PCR using Pser-up and Pser-down primers. Plasmid backbone removal was followed (Hoang et al. 2000). This construct allowed measurement of intracellular c-di-GMP levels by inference of beta-galactosidase activity (Section 2.9.3).

2.9.3 Beta-galactosidase assay

Beta-galactosidase (LacZ) activity was determined as described previously (Miller 1972; Zhang and Bremer 1995). Strains were grown on PIA medium (Section 2.2.4) for

72 h. Cells were washed twice with saline, and adjusted to an O.D of 0.7. LacZ activity was measured in 96-well microtiter plates. Five microliters of sample were added to 20 μ l of Buffer X [100 mM Na₂HPO₄, 20 mM KCl, 2 mM MgSO₄, 0.8 mg/ml of hexadecyltrimethylammonium bromide, 0.4 mg/ml of sodium deoxycholate, and 5.4 μ l/ml of β -mercaptoethanol]. To this mixture, 150 μ l of buffer Y [60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 2.7 μ l/ml of β -mercaptoethanol, and 1 mg/ml of o-nitrophenyl- β -D-galactoside (ONPG) as substrate] was added and incubated for 30 to 90 min at 30 °C. As a blank, Buffer Y minus ONPG was used for each treatment. Reactions were terminated with 175 μ l of Buffer Z (1 M sodium carbonate). Absorbance at 405 nm was measured and LacZ activities were calculated in Miller Units (Zhang and Bremer 1995).

2.10 Statistical analysis

Data were tested for normal distribution using Shapiro-Wilk test. Normally distributed data (Shapiro-Wilk test: $P > 0.05$) were analysed using ANOVA followed by an LSD Test for multiple comparison between treatments. However, data that were not normally distributed (Shapiro-Wilk test: $P < 0.05$) were analysed using non-parametrical ANOVA followed by Bonferroni (Dunn) t tests for multiple comparison between treatments. All analyses were done using SAS 9.13. Rejection level was set at $\alpha < 0.05$. Values were reported as means \pm standard error (SE).

3. Chapter Three: Results - The role of AlgL in alginate production and biofilm growth

3.1 The role of AlgL in alginate yield during biofilm mode

To investigate the role of AlgL in alginate biosynthesis, an isogenic marker-free *algL* deletion mutant was generated in the alginate over-producing strain, *P. aeruginosa* PDO300 (Section 2.4). This ‘WT’ strain is a constitutive alginate over-producer, derived by replacing the native *mucA* gene (encoding the MucA anti-sigma factor) with a variant encoding a truncated MucA22 protein. This protein is rapidly degraded, circumventing the RIP cascade (Section 1.5.1) and keeping the AlgU regulon in the ‘permanent on state’ (Mathee et al. 1999). The PDO300 Δ *algL* mutant was confirmed by colony PCR (Figure 3.1, page 76). Empty pHERD20T vectors harbouring a carbenicillin resistance marker were introduced into the WT strain and its *algL* deletion mutant as controls, while catalytically active and inactive variants of AlgL were expressed in the *algL* mutant on pHERD20T plasmids from arabinose inducible promoters (Section 2.4).

Since all strains have the plasmid-borne carbenicillin resistance marker, they could be compared under identical growth conditions. As a control experiment to show that arabinose did not have any unforeseen effects on alginate yield, I cultivated the WT strain containing an empty plasmid as a biofilm, on PIA medium containing carbenicillin in the absence and presence of arabinose. After three days’ cultivation, I measured alginate yield via an uronic acid assay (Section 2.5). As expected, the presence or absence of arabinose did not have any effect on alginate production levels in the WT strain (Figure 3.2, page 77).

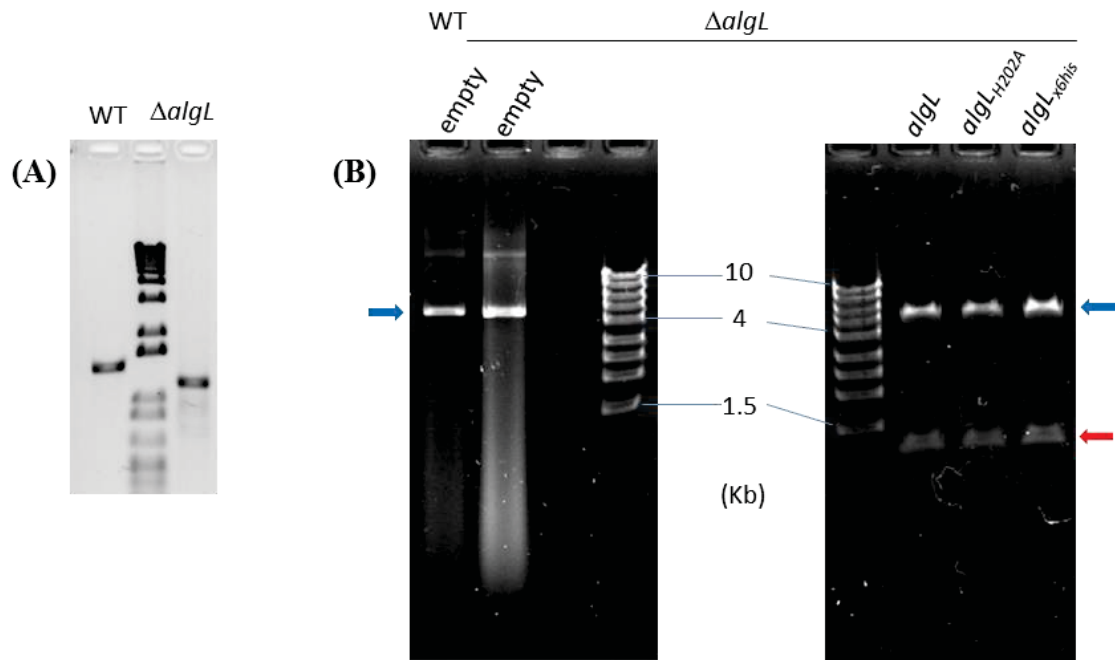


Figure 3.1 Confirmation of *algL* deletion mutant and complemented strains. (A) Colony PCR of WT strain and its *algL* deletion mutant. The centre lane is a DNA size marker (*Pst*I-Lambda) (B) *Nco*I/*Sma*I restriction digestion of plasmids isolated from the WT strain and its *algL* deletion mutant ($\Delta algL$) carrying empty vectors (empty), or $\Delta algL$ containing plasmid-borne normal *algL*, catalytically inactive (*algL*_{H202A}), or *algL* with a hexahistidine tag at its c-terminus (*algL*_{-x6his}). Blue arrow = vector backbone (5.1 kb), Red arrow = *algL* (1.1 kb). The DNA size marker included in the left and right panel of (B) is: MassRuler High Range DNA ladder (Fermentas, USA). Lane 3 of left panel is empty.

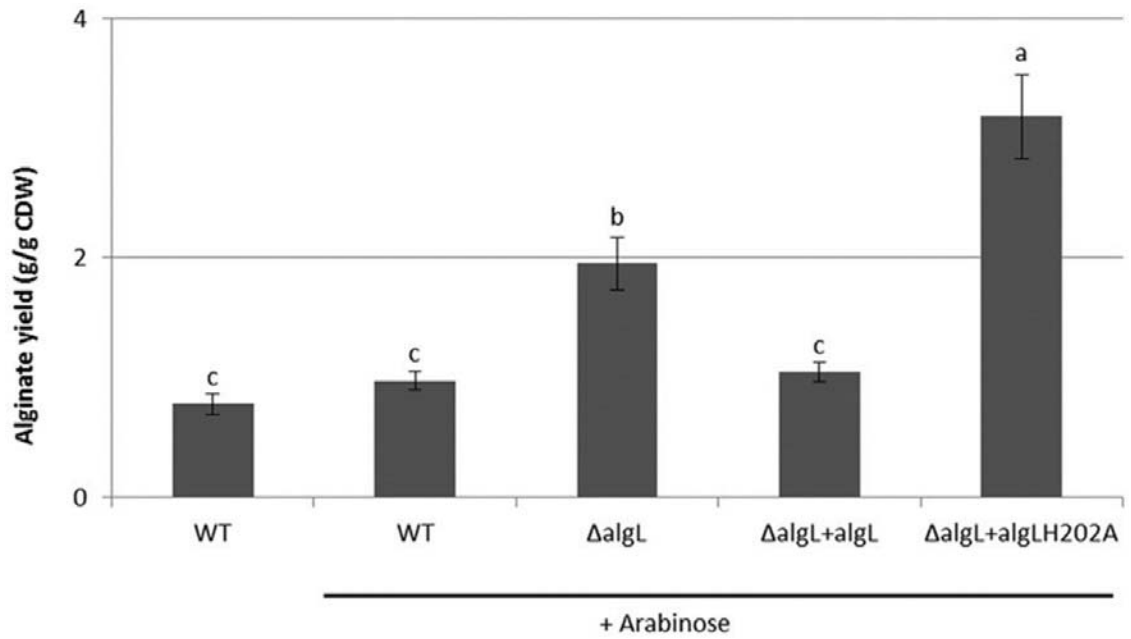


Figure 3.2 Effect of AlgL on alginate yield. Mean alginate yield \pm standard error for strains grown on PIA medium (containing 300 μ g/ml of carbenicillin) in presence (+) of arabinose inducer (0.5% w/v). Mean alginate yield is presented as grams of alginate produced per gram of cellular dry weight (g/g CDW). Different letters displayed above columns indicate significant difference ($p < 0.05$) in alginate yield across treatments as determined by ANOVA followed by an LSD Test ($n = 3$). Strains are identified as follows: WT = PDO300 containing an empty vector (pHERD20T); Δ algL, Δ algL + algL, and algL + algLH202A = *algL* deletion mutant containing an empty vector, plasmid-borne normal *algL* and catalytically inactive *algL*, respectively. Reused with permission from Nature Springer (Wang et al. 2016b).

To examine the role of AlgL in determining alginate yield, I cultured the WT strain and its *algL* deletion mutant – both harbouring empty vectors – on PIA medium containing carbenicillin and arabinose. If AlgL was important for alginate production, then deletion of *algL* would impair or stop alginate biosynthesis. Contrary to this expectation, alginate production in the *algL* deletion mutant was higher, rather than lower than in the wild type (Figure 3.2, page 77). This effect was completely reversed by reintroducing *algL* on a plasmid expressed from an arabinose inducible promoter. Thus, AlgL is likely to have a negative effect on alginate production because its absence increases alginate yield while its over-expression (in the mutant) returns alginate yield to WT levels.

To investigate if it was AlgL lyase activity that negatively affected alginate yields, I compared the alginate production levels of derivatives of the *algL* mutant expressing plasmid-borne variants of AlgL that were either catalytically active or inactive. (The inactive variant was generated by site-directed mutagenesis of its catalytic site (Albrecht and Schiller 2005; Bakkevig et al. 2005) and both strains were verified by restriction digestion (Figure 3.1, page 76)). If its lyase activity lowered alginate yield, then presence of a lyase-inactive AlgL would lead to significantly higher alginate yields than presence of catalytically active AlgL. After three days' cultivation of these strains on PIA medium containing carbenicillin and arabinose, alginate yields were measured. As predicted, the strain expressing inactive AlgL produced significantly more alginate than the strain expressing active AlgL (Figure 3.2, page 77). Since the only difference between these two strains is the presence/absence of the catalytic site of AlgL, it is reasonable to conclude that AlgL lyase activity has a direct negative effect on alginate production levels. The increase in alginate yield of the mutant variant expressing

catalytically inactive AlgL suggests that some part of the protein could be having a positive effect on alginate production.

3.2 Effect of AlgL on alginate polymer length

Because AlgL negatively affected alginate yield (Section 3.1), I suspected that it might also reduce alginate molecular weight. To test this, alginates from the WT strain and its *algL* deletion mutant – both harbouring empty pHERD20T vectors – were analysed by SEC-MALLS (Section 2.5.4). If AlgL was important for polymer length control, then deletion of *algL* would detrimentally affect its molecular weight. For instance, if AlgL is required for the production of uniform high molecular weight alginate, then deletion of *algL* may result in alginates with increased average length and greater size distribution. Theoretically, samples with a uniform molecular mass distribution should have a polydispersity index (PI) of 1.00 and indices deviating from this value signal increased sample heterogeneity (Stepito 2010). My preliminary findings showed that deletion of *algL* did not have any effect on alginate molecular weight or its size distribution (Figure 3.3, page 81), suggesting that AlgL is not important for controlling alginate polymer length.

However, since the above experiment removed AlgL and its lyase activity from the system, one could not conclude for sure whether removing only its enzymatic activity would lead to the same (or a different) effect. Therefore, I further measured and compared the average molecular masses and polydispersity indices of alginates produced by the *algL* deletion mutant expressing plasmid-borne variants of AlgL that were either catalytically active or inactive. If its lyase activity negatively affected polymer length control, then the strain over-expressing active AlgL should yield alginate of substantially lower molecular mass with greater PI value than the strain

expressing inactive AlgL. The preliminary SEC-MALLS analysis showed that while over-producing inactive AlgL did not affect alginate molecular weight and polydispersity, excess active AlgL produced alginate of much lower molecular mass and wider size distribution (Figure 3.3, page 81). Based on this result and the fact that the only difference between these strains was the presence/absence of AlgL catalytic site, excess lyase activity might detrimentally affect alginate polymer-length control. However, since knocking out *algL* alone did not have a direct effect on alginate molecular mass, it is fair to conclude that AlgL is not directly involved in polymer length control.

To rule out the possibility that excess periplasmic AlgL resulted in extracellular degradation of the polymer, I attempted to detect alginate lyase activity in the extracellular environment of biofilm. For this experiment, I grew the *algL* deletion mutant (harbouring an empty vector) and its derivative expressing plasmid-borne active AlgL on PIA medium (Section 2.2.4) containing carbenicillin and arabinose (Section 2.2.6). After three days of cultivation, biomasses were harvested and cell-free suspensions were prepared and subject to an alginate lyase plate assay (Section 2.5.6). My preliminary findings showed that over-expression of active AlgL did not lead to an increase in alginate lyase activity of cell-free biofilm suspensions (Figure 3.4, page 82), supporting previous reports that AlgL is an alginate degrading enzyme residing and exerting its activity exclusively in the periplasm.

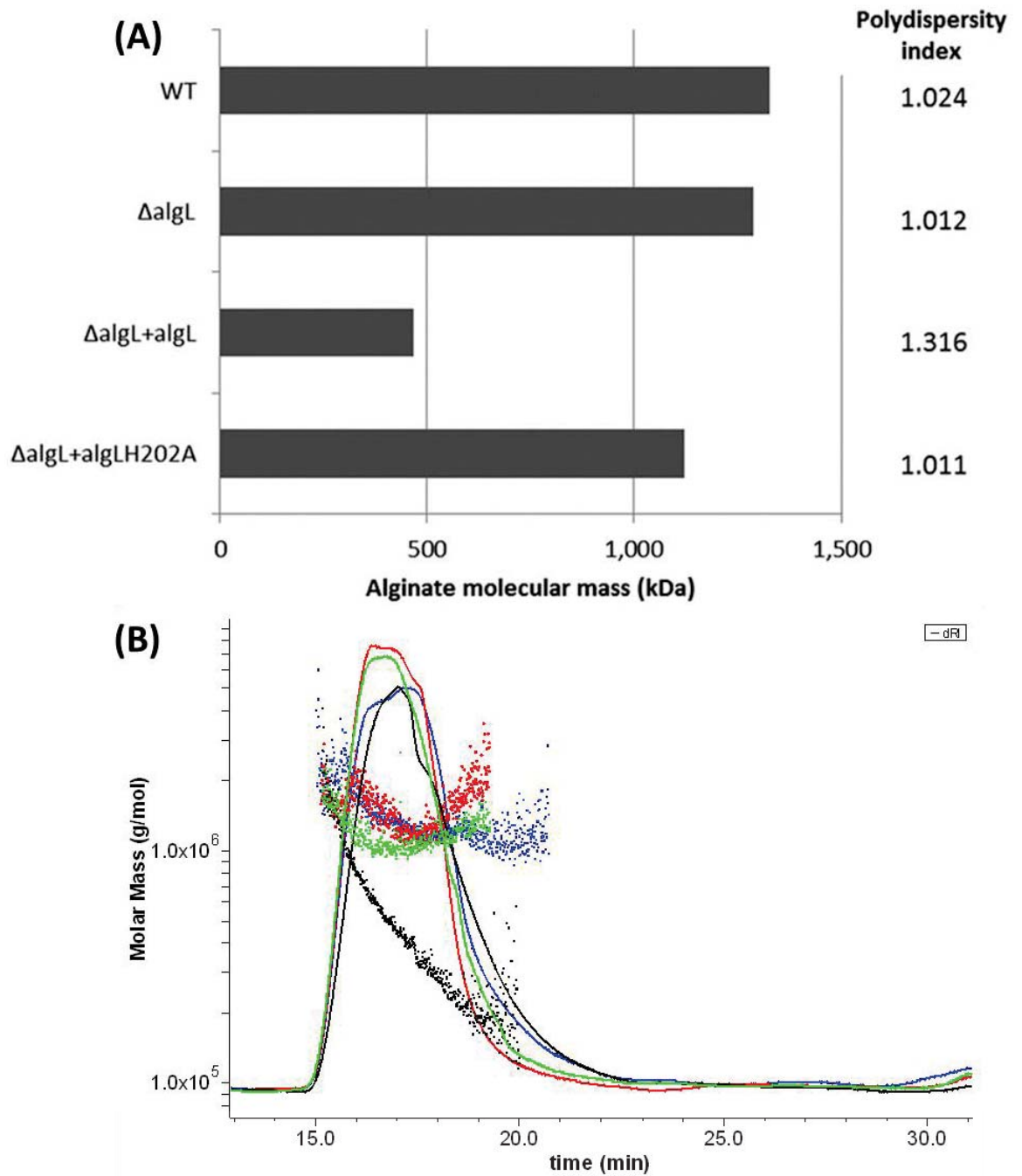


Figure 3.3 Alginate molecular weight (MW) averages and polydispersity indices as determined by SEC-MALLS. (A) Alginate samples from strains grown on PIA medium containing 300 μ g/ml of carbenicillin and 0.5% (w/v) arabinose inducer were analysed by SEC-MALLS to determine their average molecular mass and polymer length distribution (polydispersity index = PI). PI values closer to 1.0 represent a narrow MW distribution (i.e.

uniform MW). Strains are identified as follows: WT = PDO300 containing an empty vector (pHERD20T); $\Delta algL$, $\Delta algL + algL$, and $algL + algLH202A = algL$ deletion mutant containing an empty vector, plasmid-borne normal *algL* and catalytically inactive *algL*, respectively. Alginate samples were harvested from six plates for each strain and then pooled for MW analysis. Since this experiment was only done once, its result can only be considered as preliminary. Graphic output of SEC-MALLS analysis is shown panel (B). Panel (A) reused with permission from Nature Springer (Wang et al. 2016b).

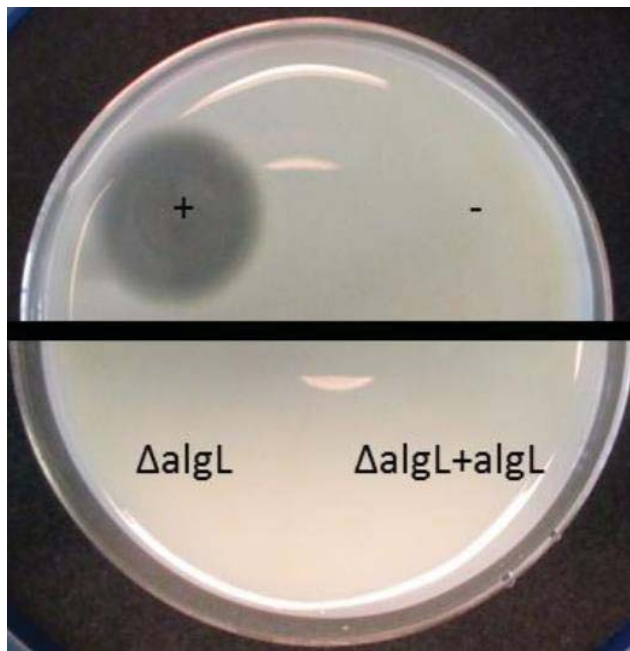


Figure 3.4 Alginate lyase assay of strains grown on solid media. Shown are alginase assay plates incubated for 24 h at 37 °C then flooded overnight with 10% w/v cetylpyridinium chloride solution. *Top-panel:* Commercial alginate lyase (Sigma Aldrich, USA) and autoclaved saline were used as positive (+) and negative (-) controls. *Bottom-panel:* 100 μ L of cell-free resuspensions of biofilms grown on PIA medium. Samples were from the *algL* deletion mutant containing an empty vector ($\Delta algL$) and *algL* deletion mutant expressing plasmid-borne *algL* ($\Delta algL+algL$). Clearings indicate alginate degradation. Since this experiment was only done once, its result can only be considered as preliminary.

3.3 Effect of AlgL on alginate composition

As noted earlier, expression of *algL in trans* in the *algL* deletion mutant did not restore all phenotypes (i.e. polymer length) to WT levels. One of these phenotypes was O-acetylation. To examine the effect of AlgL on alginate composition, alginates produced by the WT strain and its *algL* deletion mutant – both harbouring empty vectors - were analysed by ¹H-NMR (Section 2.5.5). Bacteria were grown on PIA medium and alginate from six plates for each strain were harvested and pooled for composition determination. My preliminary findings showed that deletion of *algL* did not affect epimerization degree, suggesting that AlgL might not have a direct role in controlling alginate composition.

However, as alluded to above, the deletion was associated with a substantial drop in O-acetylation levels (WT ~70% vs *algL* deletion mutant ~20%) and subsequent complementation (by either expressing plasmid-borne active or inactive variants of AlgL in the *algL* deletion mutant) could not restore O-acetylation to WT levels (Table 3.1, page 84). This preliminary result suggests that deletion of *algL* gene may have caused polar effects on downstream genes involved in O-acetylation (*algI*, *J* and *F*). Hence, additional experiments (e.g. RT-PCR or immunoblotting) could be done in the future to see if the *algL* mutation caused any polar effects on downstream genes.

TABLE 3.1 Alginate composition as measured by ¹H-NMR.

Strain	F _G	G _M	F _{GM/MG}	F _{MM}	F _{GG}	AC%
WT	0.28	0.72	0.28	0.44	0	70
ΔalgL	0.35	0.65	0.35	0.30	0	17
ΔalgL + algL	0.36	0.64	0.36	0.28	0	20
ΔalgL + algLH202A	0.36	0.64	0.36	0.28	0	19

Alginate samples, obtained from strains grown on PIA medium containing carbenicillin (300 μg/ml) and arabinose inducer at 0.5% (w/v), were analysed by ¹H-NMR to investigate their level of epimerization and O-acetylation. F_G = mole fraction of guluronate residues in alginate chain. F_M = mole fraction of mannuronate residues in alginate chain. F_{GM/MG}, F_{MM} and F_{GG} = mole fraction of GM/MG, MM and GG diads. AC% = degree of O-acetylation. Strains are identified as follows: WT = PDO300 containing an empty vector (pHERD20T); ΔalgL, ΔalgL + algL, and algL + algLH202A = *algL* deletion mutant containing an empty vector, plasmid-borne normal *algL* and catalytically inactive *algL*, respectively. Alginate samples were harvested from six plates for each strain and then pooled for compositional analysis. Since this experiment was only done once, its result can only be considered as preliminary. Table Reused with permission from Nature Springer (Wang et al. 2016b).

3.4 Role of AlgL in stability of biosynthesis complex

Since deletion of *algL* did not appear to have compromised alginate yield (Section 3.1), alginate molecular weight control (Section 3.2) or composition determination (Section 3.3), this gene might not be essential for the function or stability of the alginate biosynthesis apparatus. To test this, I probed membrane fractions with various antibodies to determine whether deletion of *algL* had any effect on the stability of structural components of the complex. The WT strain and its *algL* deletion mutant were grown on PIA medium for 72 hrs. After three days' incubation, cells were harvested, lysed and envelope fractions were prepared (Section 2.6.1 & 2.6.2) and subjected to SDS-PAGE (Section 2.6.6) and immunoblot analysis (Section 2.6.7). If AlgL was important for the stability of the alginate biosynthesis apparatus, then deletion of *algL* would destabilise structural components. Deletion of *algL* did not affect the stability of Alg44, AlgK, AlgX or AlgE (Figure 3.5, lanes 5 & 6 of each panel, page 86), implying that AlgL might not be critical for the stability of the complex. However, since these experiments did not include the complemented strains as controls, these results should only be treated as preliminary.

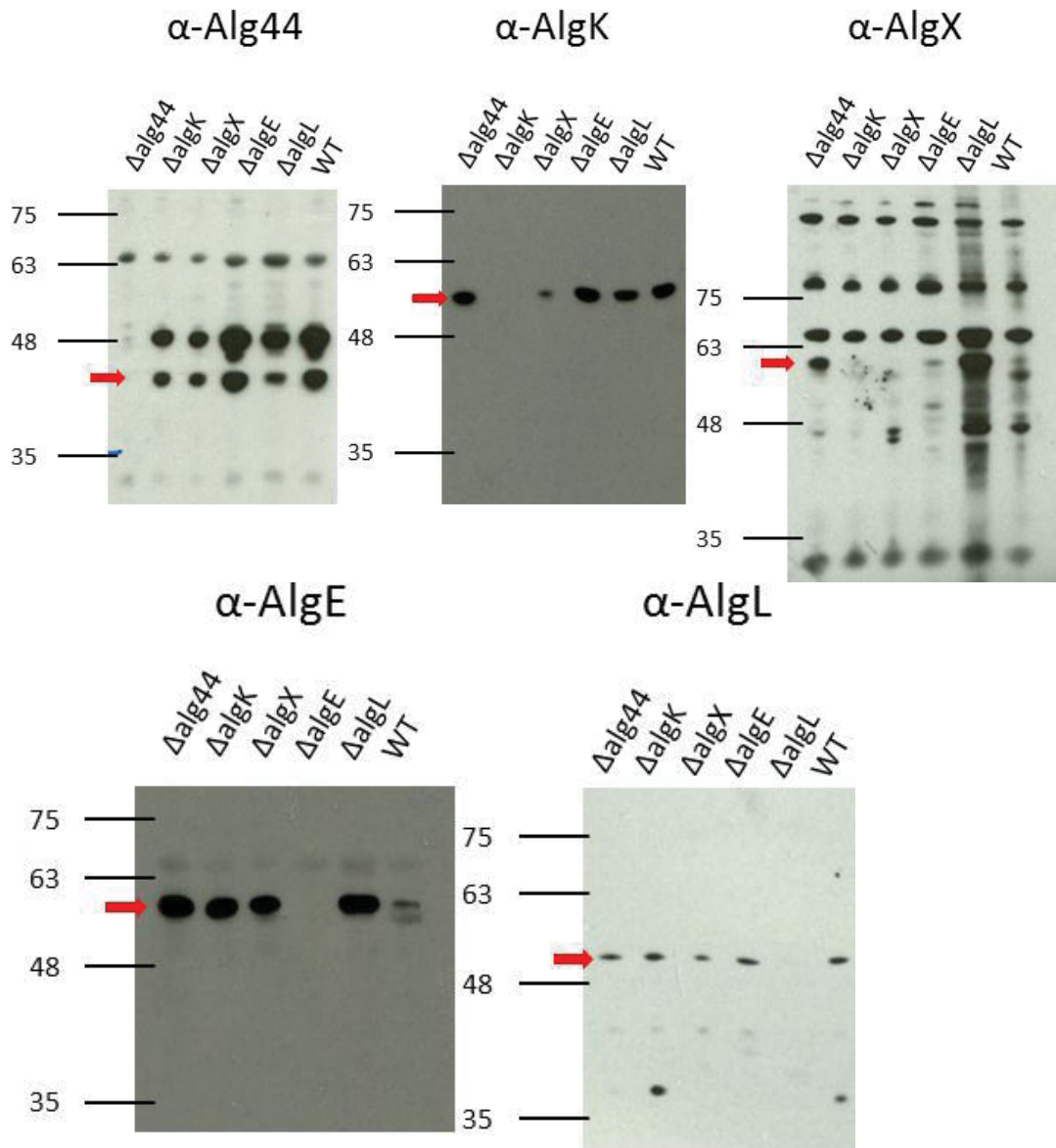


Figure 3.5 Immunoblots for detection of components of the alginate biosynthesis apparatus in various alg mutants. Shown are immunoblots of envelope fractions of various strains using anti-Alg antibodies (top row, from left: α -Alg44, α -AlgK, α -AlgX; bottom row, from left: α -AlgE and α -AlgL) to detect specific components of biosynthesis complex. Red arrows highlight respective target proteins at expected molecular weight. Strains are identified as follows: Δ alg44 = PDO300 Δ alg44, Δ algK = PDO300 Δ algK, Δ algX = PDO300 Δ algX, Δ algE = PDO300 Δ algE, Δ algL = PDO300 Δ algL, and WT = *P. aeruginosa* PDO300. MW markers are shown in kDa.

If AlgL is not required for the stability of the complex, then deletion of other subunits might also not affect AlgL stability. To test this, I cultured previously generated (Gutsche et al. 2006; Hay et al. 2010b; Rehman et al. 2013; Remminghorst and Rehm 2006a) mutants of the WT strain lacking specific subunits (PDO300 Δ *alg44*, PDO300 Δ *algK*, PDO300 Δ *algX* and PDO300 Δ *algE*) as above, and prepared and probed membrane fractions with anti-AlgL (and other) antibodies. My results show that while deletion of other subunits compromised alginate production and stability of some other structural components, AlgL remained stable (Figure 3.5, lanes 1-4 of each panel, page 86), suggesting that AlgL could be a free periplasmic protein that is not essential for the stability or function of the biosynthesis apparatus. However, in light of previously encountered complementation issues (Sections 3.2 and 3.3) and the incomplete nature of these experiments (i.e. lack of properly complementing strains), these results can only be considered as preliminary.

3.5 Pull-down and immunoblot experiments to identify AlgL interaction partners

Results thus far imply that AlgL is not essential for the function (Sections 3.1, 3.2 and 3.3) or stability (Section 3.4) of the biosynthesis apparatus. If this is the case, then AlgL would not be expected to interact with other subunits of the apparatus. To test this, I performed pull down and immunoblot experiments by expressing plasmid-borne *algL* gene, encoding AlgL protein with a c-terminal hexahistidine tag, from an arabinose inducible promoter in the *algL* deletion mutant (which was confirmed by plasmid isolation and restriction digestion (Figure 3.1B, page 76)). This strain was grown on PIA medium (Section 2.2.4) containing carbenicillin (Section 2.2.6) and arabinose for three days, after which, envelope fractions were prepared and solubilized in Buffer C (Section 2.6.5). Solubilized samples were subject to NIAC purification (Section 2.6.5). After several washing steps to remove impurities and non-specifically interacting-proteins, samples were eluted in elution buffer, ran on SDS-PAGE (Section 2.6.6), transferred to nitrocellulose membrane and subjected to immunoblot analysis (Section 2.6.7). If AlgL was a free periplasmic protein, then none of the other subunits should be detectable (by immunoblot) in the eluted fractions from pull down experiments. When probed with various antibodies, none of Alg44, K, X and G was detected in eluted fractions (Figure 3.6, page 89), lending further support to the preliminary data presented above (Section 3.4), alluding to the possibility that AlgL could be a free periplasmic protein.

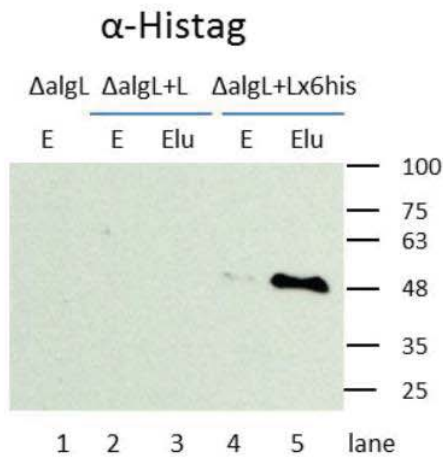
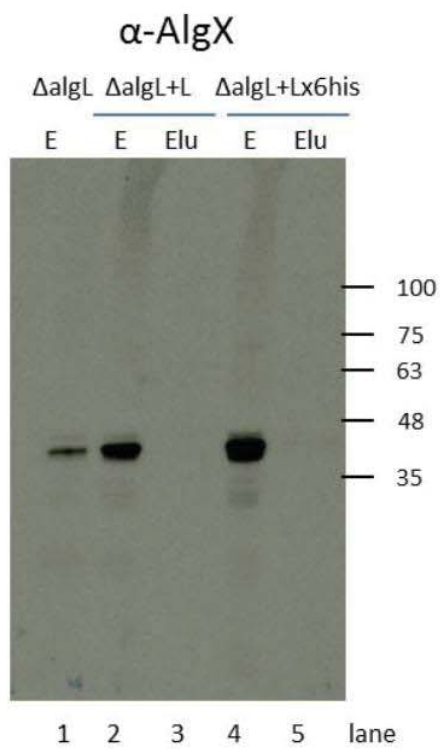
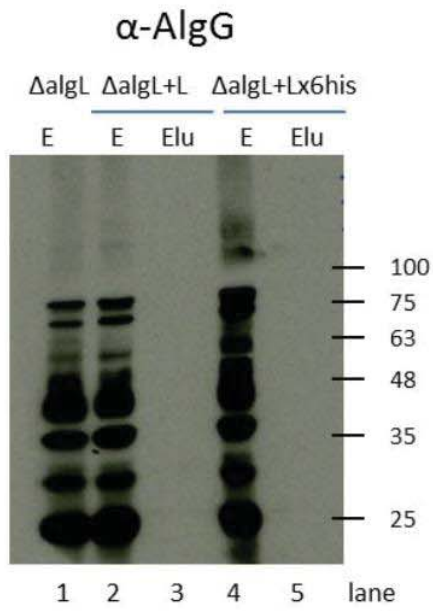
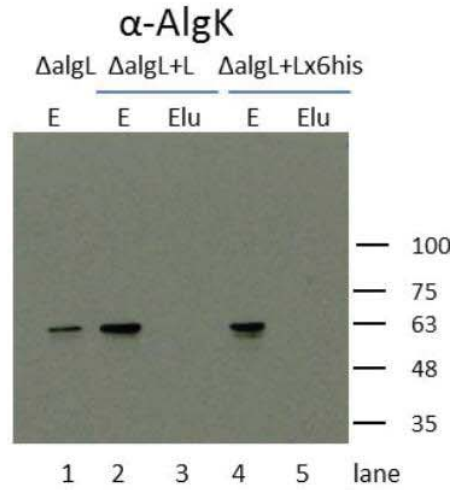
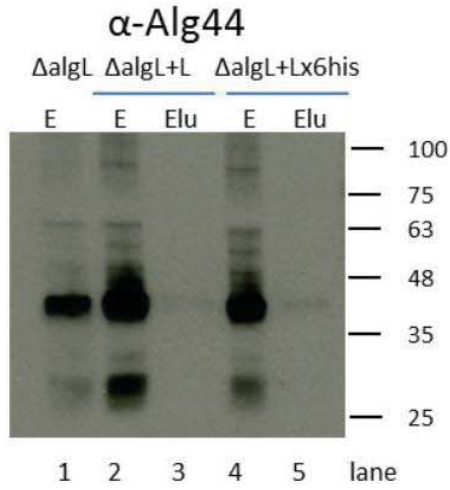


Figure 3.6 Immunoblots showing the detection of hexahistidine-tagged AlgL and its interaction partners. Strains were grown on PIA medium supplemented with 300 $\mu\text{g/ml}$ of carbenicillin (except for PDO300 ΔalgL , lane 1) and 0.05% (w/v) arabinose for 72 h at 37 °C. Whole Envelope (E) fractions were prepared and histagged-AlgL and its co-interacting proteins were purified with Complete His-Tag Purification Resin (Roche). Envelope (E) and eluted (Elu) fractions were run on SDS-PAGE, transferred to nitrocellulose membrane and subjected to immunoblot with various antibodies (identified above each panel: α -Alg44, α -AlgK, α -AlgG, α -AlgX and α -Histag), as described in methods section. In each panel, Lane 1 = Envelope fraction of PDO300 ΔalgL . Lanes 2 and 3 = Whole Envelope (E) and eluted (Elu) fractions of PDO300 ΔalgL (pHERD20T:*algL*), respectively. Lanes 4 and 5 = Whole Envelope (E) and eluted (Elu) fractions of PDO300 ΔalgL (pHERD20T *algLx6his*), respectively. Molecular weight of protein standards are shown in kDa.

3.6 Effect of AlgL on cell attachment, biofilm growth and dispersal

In earlier sections, it was suggested that AlgL negatively affected alginate yield (Section 3.1), without directly affecting alginate polymer length or composition (Section 3.2 & 3.3), and that AlgL is not an essential player (or component) of the alginate biosynthesis apparatus (Section 3.4 & 3.5). Since AlgL is an alginate lyase and alginate makes up a substantial proportion of *P. aeruginosa* PDO300 biofilms, it seemed reasonable to hypothesize that excess AlgL activity would increase polymer degradation, potentially affecting biofilm attachment, growth and dispersal.

To test this, I assessed the WT strain and its *algL* deletion mutant – both harbouring empty pHERD20T vectors – along with the mutant over-expressing plasmid-borne AlgL, using a 96-well plate biofilm assay (Section 2.8.1). If AlgL was important for attachment, then deletion of *algL* would reduce attachment efficiency. While deletion of *algL* reduced attachment, expressing the gene from a plasmid did not restore it to WT levels (Figure 3.7A, page 92), suggesting that AlgL does not play a role in attachment in the deletion mutant background.

Subsequent analysis of biofilm growth after 72 hrs of incubation showed no effect of AlgL copy number (or alginate yield, molecular mass or composition) on biofilm biomass (Figure 3.7B, page 92). However, although dispersal was increased in the deletion mutant (Figure 3.7C, page 92), reintroducing the gene on a plasmid did not restore dispersal to WT levels.

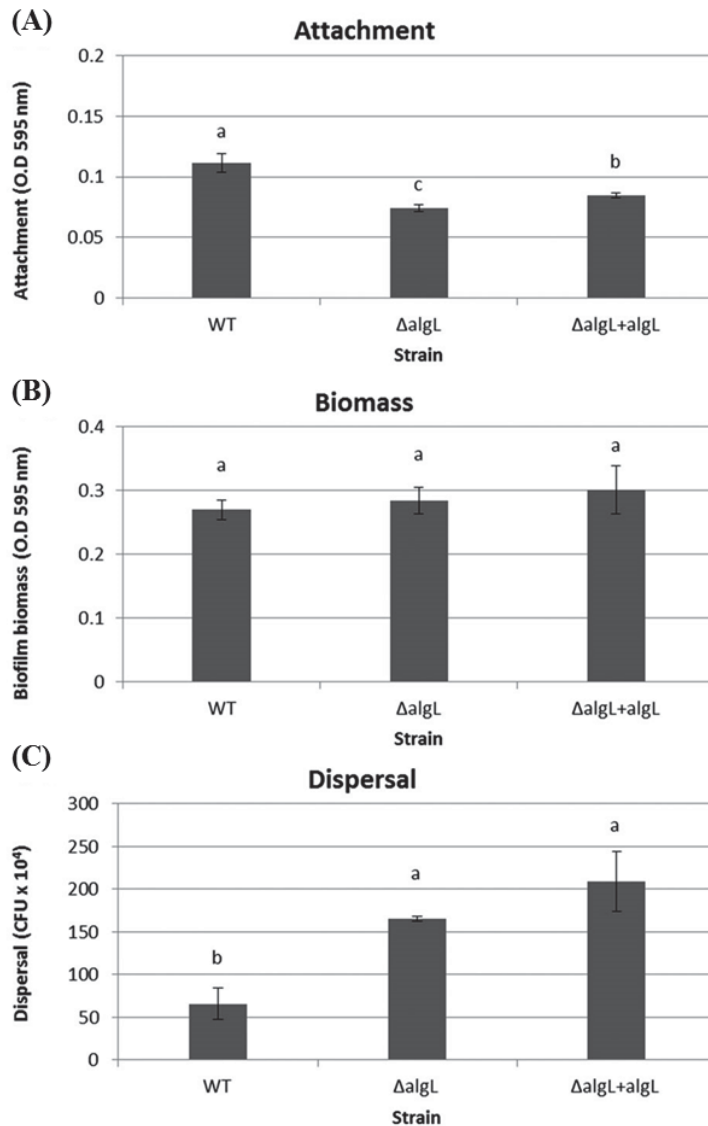


Figure 3.7 Effect of AlgL on cell attachment, biofilm biomass and dispersal. These biofilm phenotypes were analysed in a microtiter plate assay. **(A)** Mean attachment efficiencies \pm SE ($n = 8$) at 2 h. **(B)** Mean biomass of biofilms \pm SE ($n = 4$) at 72 h. Attachment and biomass was expressed as absorbance at 595 nm, as determined by crystal violet staining. **(C)** Mean dispersal efficiencies \pm SE ($n = 4$) at 72 h expressed as colony forming units (CFU $\times 10^4$ per mL). Different letters displayed above columns indicate statistically significant differences ($p < 0.05$). Attachment efficiencies **(A)** were not normally distributed (Shapiro-Wilk test: $P < 0.05$), thus

analysed by non-parametrical ANOVA followed by Bonferroni (Dunn) t tests for multiple comparison between treatments. Biofilm biomasses (**B**) and dispersal efficiencies (**C**) were normally distributed (Shapiro-Wilk test: $P > 0.05$), thus analysed by ANOVA with an LSD Test for multiple comparison between treatments. Strains are identified as follows: WT = PDO300(pHERD20T), $\Delta algL$ = PDO300 $\Delta algL$ (pHERD20T), and $\Delta algL + algL$ = PDO300 $\Delta algL$ (pHERD20T:*algL*). Reused with permission from Nature Springer (Wang et al. 2016b).

3.7 Effect of O-acetylation on cell attachment, biofilm growth and dispersal

Even though plasmid-borne expression of *algL* did not restore O-acetylation (Section 3.3), attachment or dispersal (Section 3.6) to WT levels, the apparent correlation between O-acetylation with attachment and dispersal raised the tentative possibility that O-acetylation influences these phenotypes. To examine if O-acetylation levels truly influenced attachment and dispersal, I conducted an experiment using two previously described strains that produced o-acetylated (~10% AC) and non-o-acetylated alginate of similar yield, molecular weight and epimerization degree. These two strains generated by Moradali et al (2015) were subjected to the same 96 well plate assays (excluding arabinose while substituting carbenicillin for gentamicin) (Section 2.8.1). I hypothesized that if O-acetylation degree directly determined attachment and dispersal efficiency, then a complete loss of this modification would influence both of these phenotypes. Although abolishing O-acetylation did not affect biofilm growth (Figure 3.8B, page 94), it impaired both attachment (Figure 3.8A, page 94) and dispersal (Figure 3.8C, page 94).

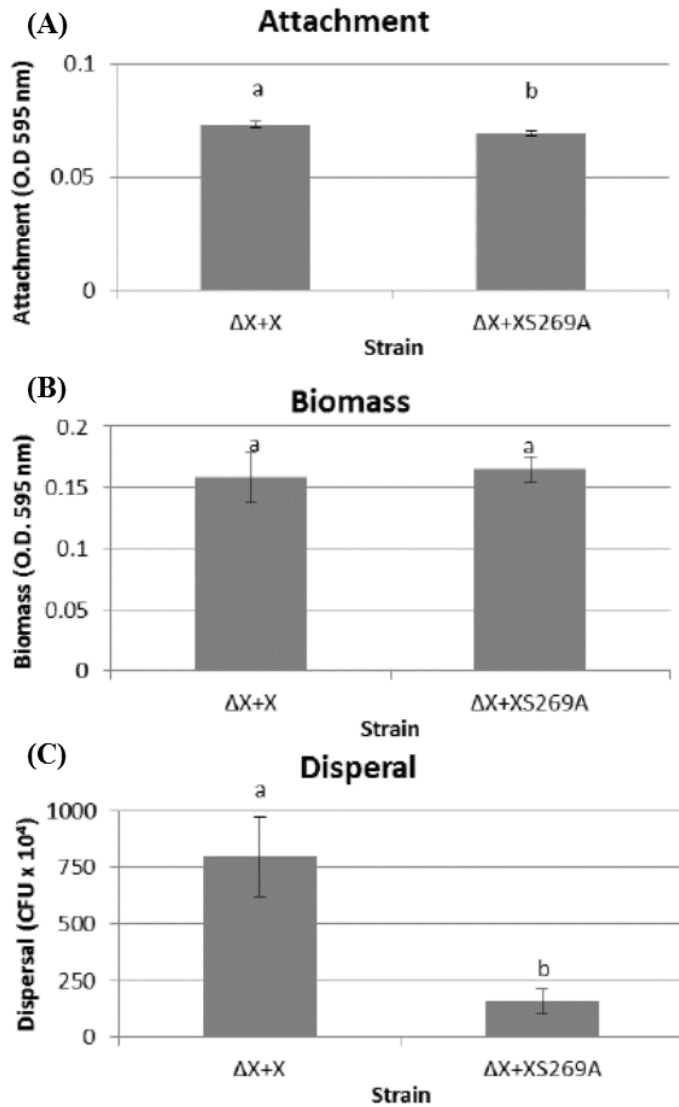


Figure 3.8 Effect of alginate O-acetylation on cell attachment, biofilm biomass and dispersal efficiency. Attachment efficiencies, biofilm biomasses and dispersal efficiencies were analysed in a microtiter plate assay. **(A)** Mean attachment efficiencies \pm SE (n = 6) at 2 h, as determined by crystal violet staining. Attachment efficiencies are expressed as absorbances at 595 nm. **(B)** Mean biomass of biofilms \pm SE (n = 4) at 72 h, as determined by crystal violet staining. **(C)** Mean dispersal efficiencies \pm SE (n = 6) at 72 h expressed as colony forming units (CFU x 10⁴ per mL). In panels **(A-C)** different letters displayed above columns indicate statistically significant differences (two-way, t-test, p < 0.05) in attachment efficiency, biofilm

biomass and dispersal efficiency, respectively. In panels (A-C), strains $\Delta algX+algX$ and $\Delta algL+algXS269A$ are identified as PDO300 $\Delta algX$ (pBBR1MCS-5:*algX*) and PDO300 $\Delta algX$ (pBBR1MCS-5:*algXS269A*), respectively. These strains produce o-acetylated and non-acetylated forms of alginate, respectively.

4. Chapter Four: Results - Insight into the functions of MucR in the regulation of alginate production

In this part of the study I investigated the biological function of MucR, an inner membrane protein thought to be a chief driver of alginate production. Current models suggest that it provides a local pool of c-di-GMP driving biosynthesis at a post-translational level (Hay et al. 2009b). This protein has a membrane-embedded sensor domain and two cytoplasmic output domains involved in c-di-GMP turnover. However, whether both sensor and output domains are crucial for making alginate is unknown. Here I explored the role of MucR, with special reference to its output and sensor domains and its response to nitrate.

The two cytoplasmic output domains of MucR, diguanylate cyclase (DGC) and phosphodiesterase (PDE), have been shown *in vitro* to make and break c-di-GMP, respectively (Li et al. 2013). *In vitro* studies using a truncated version of MucR, consisting of only its output domains, revealed that the DGC activity was enhanced by its PDE domain, suggesting that both contribute to intracellular c-di-GMP levels (Li et al. 2013). However, the physiological relevance of this *in vitro* observation is currently unclear, since normally the sensor domain is at the N-terminus of the protein, which could regulate the activity of its output domains *in vivo*.

The sensor domain of MucR contains three MHYT motifs thought to co-ordinate a copper ion for the perception of a signal (Galperin et al. 2001). A likely signal could be nitrate because *P. aeruginosa* uses it in denitrification, releasing intermediates that are linked to reduced alginate yields (Wood et al. 2007; Zumft 1997). One of these intermediates, nitric oxide, also enhances biofilm dispersal through MucR by lowering global c-di-GMP levels (Li et al. 2013). However, it is still not clear whether nitrate

could be a signal perceived by MucR, leading to reduced alginate yields from cells cultivated in biofilm and planktonic growth modes.

Since elevated c-di-GMP levels up-regulate genes involved in biofilm formation (Jenal et al. 2017), MucR and its putative signal (nitrate) could also affect alginate production at a gene-expression level through modulation of global c-di-GMP levels. I also postulated that any effect of MucR and its signal on c-di-GMP levels would also affect other phenotypes sensitive to this ubiquitous bacterial secondary messenger - including swarming motility, attachment, polysaccharide production and biofilm formation.

I carried out a series of experiments to explore the functions of MucR in the regulation of alginate. Figure 4.1 (page 98) summarizes the proposed model that was explored in this study for the function of MucR and nitrate in alginate production, modulation of c-di-GMP and c-di-GMP sensitive phenotypes.

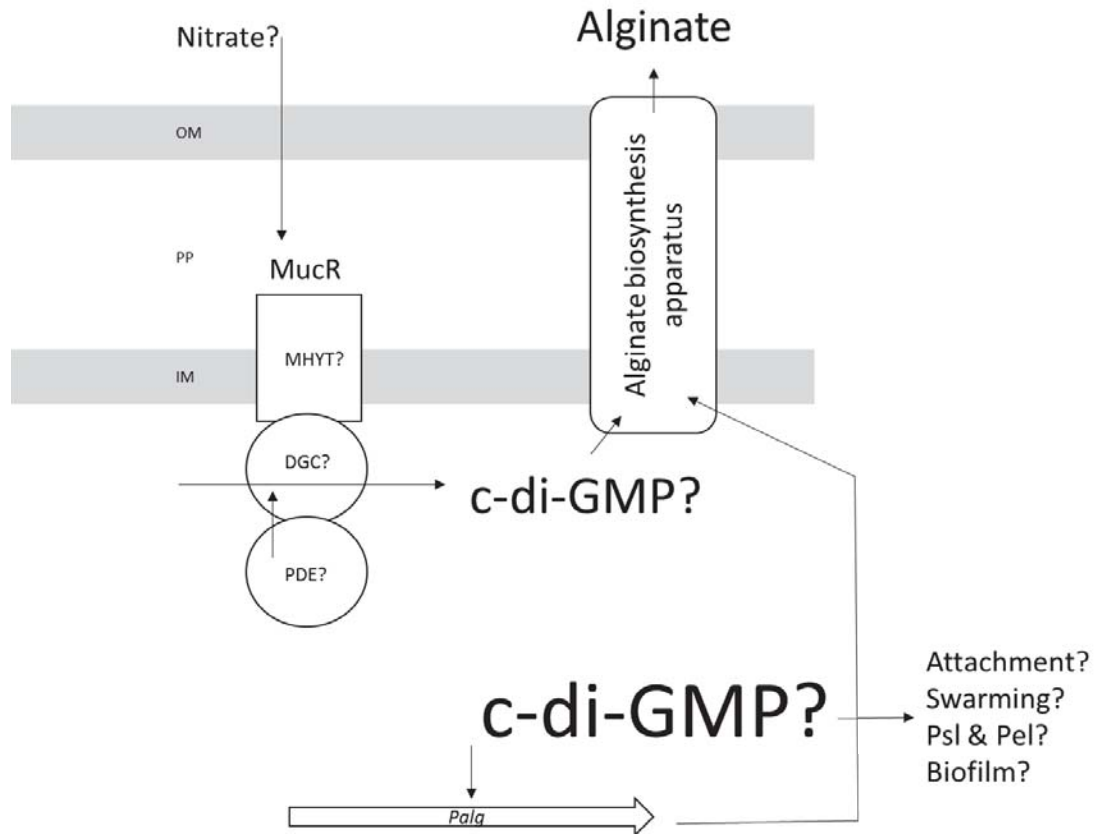


Figure 4.1 Overview of the proposed working model for the function of nitrate and MucR in alginate production, c-di-GMP levels and other phenotypes sensitive to c-di-GMP. A signal (nitrate?) is perceived by MucR (via its MHYT sensor domain?), affecting the activity of its output domain (DGC and PDE?) involved in c-di-GMP turn-over. A local pool (global?) of c-di-GMP imparted by MucR presumably drives alginate production at a post-translational (or a transcriptional level?). The effect of nitrate and MucR on c-di-GMP levels may impact other phenotypes including attachment, swarming, Psl & Pel production, biofilm formation which are sensitive to this secondary messenger molecule.

4.1 MucR's DGC and PDE domains are important for alginate biosynthesis

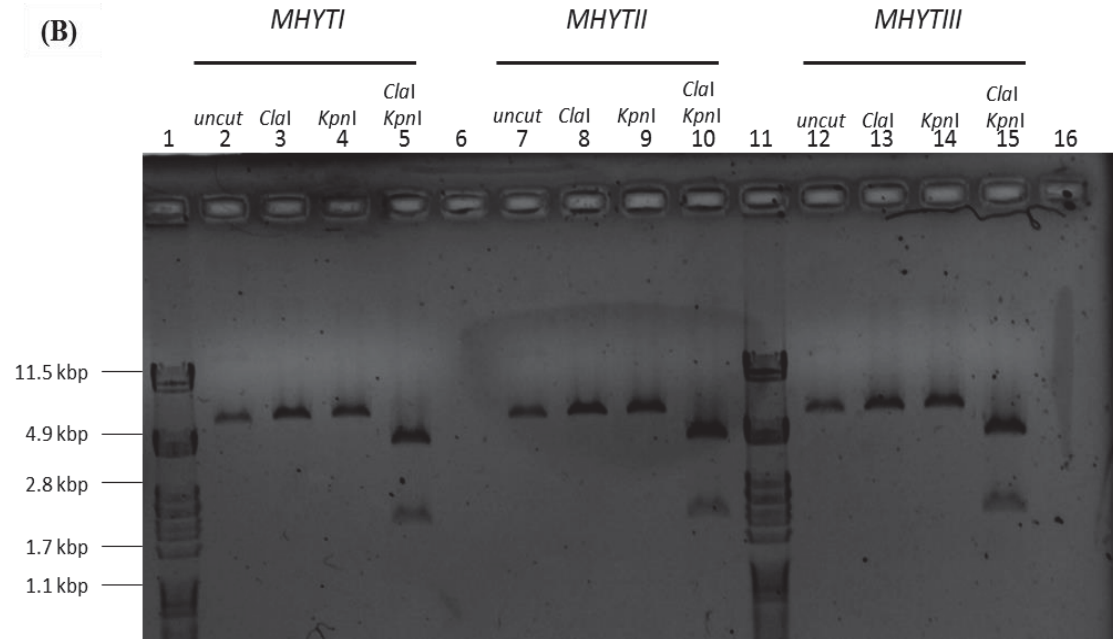
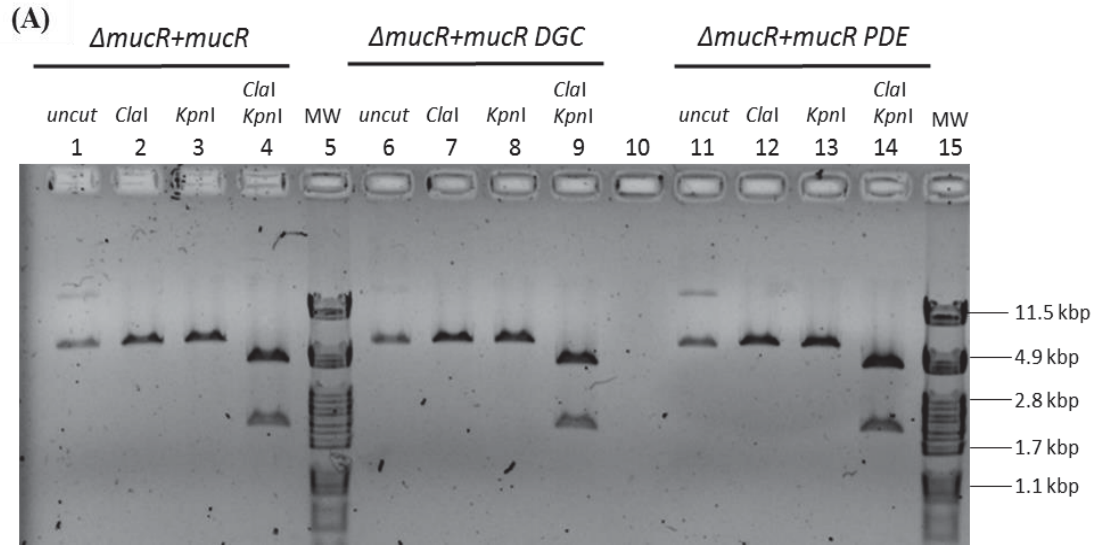
Some preliminary data suggested that *mucR* was important for alginate biosynthesis (Hay et al. 2009b) as deletion of *mucR* caused a substantial reduction in alginate yield. Furthermore, plasmid-borne over-expression of MucR significantly increased alginate production (Hay et al. 2009b). However, when the protein was truncated (i.e. both DGC and PDE domains were removed), this effect did not occur (Hay et al. 2009b). Since *in vitro* studies have shown that the output domains of MucR are involved in c-di-GMP turnover, with one facilitating its synthesis and the other its degradation (Li et al. 2013), I assumed that the failure of the truncated version of MucR to increase alginate yields was due to the complete lack of domains involved in c-di-GMP turnover. If this hypothesis is correct, then eliminating MucR's c-di-GMP turnover activity by introducing point mutations into each of the catalytic residues of MucR's output domains would stop it from promoting alginate biosynthesis.

To test this postulation, I expressed variants of MucR with mutated DGC and PDE domains from constitutive *lacZ* promoters on a medium copy number plasmid (pBBR1MCS-5) in a mutant where the native *mucR* gene had been deleted by homologous recombination (Hay et al. 2009b). These variants were constructed and cloned into pBBR1MCS-5, and DGC and PDE active sites disabled by alanine substitution (GGDEF to GGAAF and EAL to AAL, respectively) (Wang et al. 2015). Such mutations were expected to disable the output domains of MucR, preventing it from synthesising and degrading c-di-GMP, respectively.

These specific mutations were introduced because when such changes were made to other enzymes involved in c-di-GMP production/degradation, enzymatic activities were lost (reviewed in (Jenal et al. 2017)). Variant-containing plasmids were verified by DNA sequencing (Section 2.3.9) then introduced into *E. coli* S17-1 (Section 2.3.10) for

conjugation into the *mucR* deletion mutant (Section 2.3.11). Potential transconjugants were selected for on PIA medium containing gentamicin. Received plasmids were isolated and subjected to restriction digestion confirmation (Figure 4.2A, page 101). Variants of the *mucR* deletion mutant harbouring either an empty vector or a plasmid-borne *mucR* were included, along with the WT strain containing an “empty” plasmid (i.e the cloning vector pBBR1MCS-5 containing the selectable gentamicin resistance cassette). The presence of vector-borne gentamicin resistance markers in all strains allowed me to compare the performance of different strains (and versions of MucR) under identical growth conditions.

To determine the role of MucR’s DGC and PDE domains in alginate production during biofilm growth, the above strains – *mucR* deletion mutant with: empty vector, plasmid-borne MucR, plasmid-borne MucR with a defunct DGC domain, and plasmid-borne MucR with a defunct PDE domain — were grown on PIA medium containing gentamicin for 72 h, along with the WT strain containing an empty vector as a control. Biomass was harvested and alginate samples prepared and quantified by an uronic acid assay. If DGC and PDE domains of MucR enhanced alginate production, then plasmid-borne expression of functional MucR would increase while expression of defunct MucR would decrease (or not affect) alginate yields. My findings support this prediction, i.e. expression of functional MucR from a plasmid increased alginate production levels and plasmid-borne expression of defunct MucR failed to increase alginate yields (Figure 4.3, page 103).



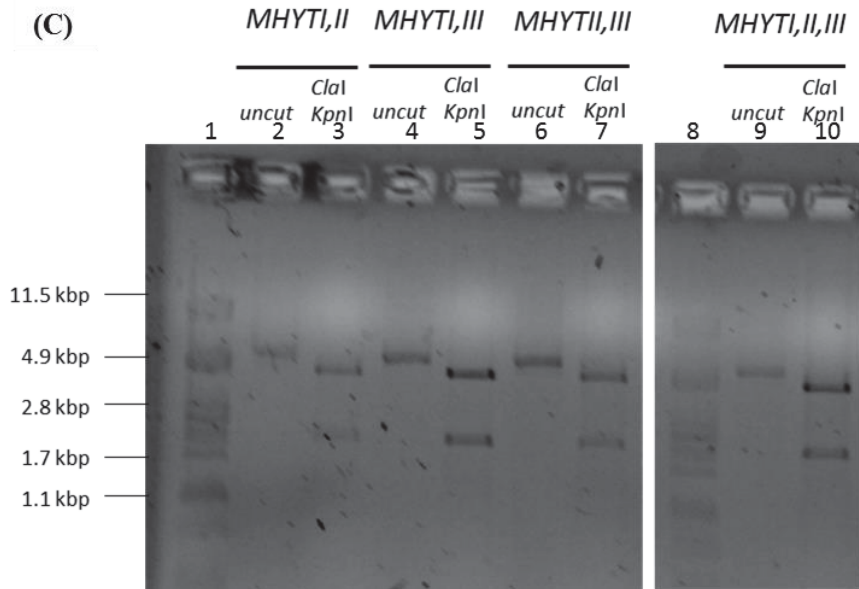


Figure 4.2 Agarose gel electrophoresis of restriction digests to confirm plasmid DNA isolated from PDO300 Δ *mucR* trans-conjugants receiving pBBR1MCS-5 vectors harbour variants of *mucR* gene. In panels (A) and (B), Plasmid DNA isolated from each strain were (from left-to-right) not digested (uncut: lanes 1, 6, 11), cut with *ClaI* (lanes 2, 7, 12), cut with *KpnI* (lanes 3, 8, 13) or cut with both *ClaI* and *KpnI* (lanes 4, 9, 14). As expected, plasmids cut with either *ClaI* or *KpnI* produced one band ~6.8 kbp in size. In panel (C), Plasmid DNA was either uncut or cut with both *ClaI* and *KpnI*. In all panels, samples digested with both *ClaI* and *KpnI* produced two DNA fragments of ~4.7 (linearized vector) and ~2.1 kbp (variant of *mucR* gene). A DNA size marker (*PstI*-lambda) was included in lanes 5 & 15 of panel (A), lanes 1 & 11 of panel (B), and lanes 1 & 8 of panel (C).

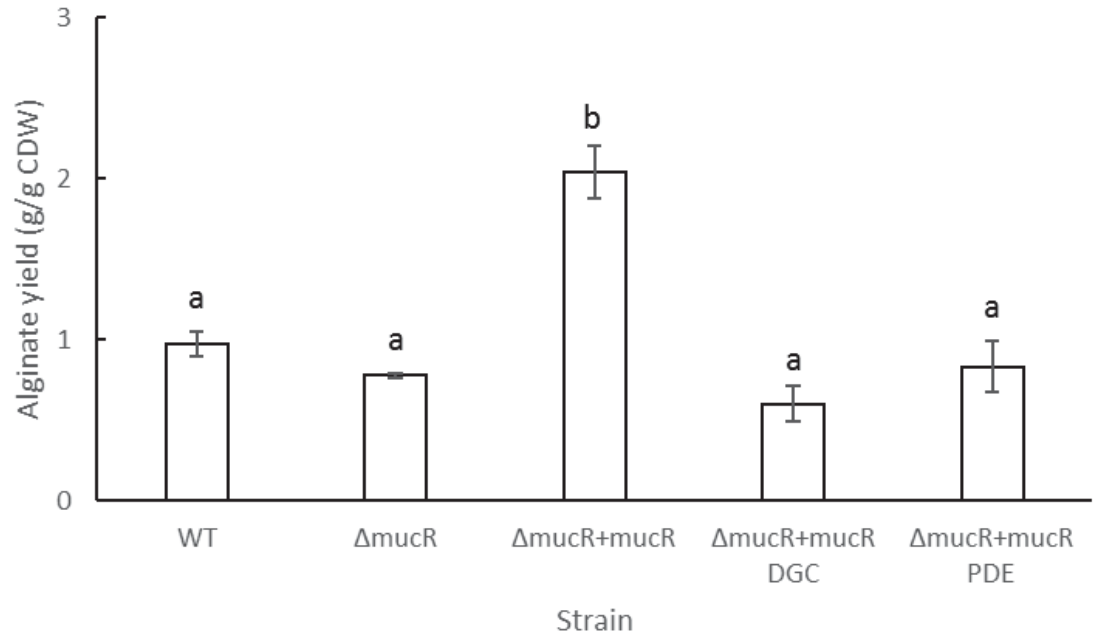


Figure 4.3 Effect on alginate yield of deleting *mucR* and plasmid-borne expression of *mucR* and variants with mutated DGC and PDE domains during biofilm growth. Shown are mean alginate yields \pm SE, representative of three independent experiments. The strains tested were: WT (PDO300; containing an empty pBBR1MCS-5 plasmid), a *mucR* deletion mutant ($\Delta mucR$; containing an empty pBBR1MCS-5), a *mucR* deletion mutant expressing plasmid-borne *mucR* ($\Delta mucR+mucR$), and a *mucR* deletion mutant expressing variants of *mucR* encoding MucR with inactive DGC or PDE domains ($\Delta mucR+mucR$ DGC and $\Delta mucR+mucR$ PDE, respectively). Alginate yields labelled with different letters differed significantly from each other (LSD Test, $p < 0.05$).

To exclude alternative possibilities that these mutations destabilized MucR, leading to its degradation, inner membrane fractions of cells were prepared and probed with anti-MucR antibodies. Cells were grown as biofilms on PIA medium, harvested, lysed, and inner membrane fractions were isolated (Section 2.6.2), protein concentrations measured (Section 2.6.3), samples ran on SDS-PAGE (Section 2.6.6) and analysed by immunoblot (Sections 2.6.7). My findings show that deletion of *mucR* gene abolished the presence of the MucR protein (see Figure 4.4, lanes 3 & 4, page 105; red arrow showing expected size of *mucR* gene) but inactivation of either DGC or PDE domain did not jeopardize MucR stability (Figure 4.4, lanes 13-16, page 105).

My results thus strongly suggest that both DGC and PDE domains of MucR are important for alginate production (Figure 4.5, page 106). This may be attributed to their roles in making and breaking c-di-GMP, as demonstrated *in vitro* (Li et al. 2013). Interestingly, it was previously reported that deletion of *mucR* reduced alginate production (Hay et al. 2009) but my current work demonstrates that alginate yields of the WT strain and its *mucR* deletion mutant were not significantly different (Figure 4.3, page 103). The variance of findings between these two studies could result from different experimental design (see discussion, Section 5.2).

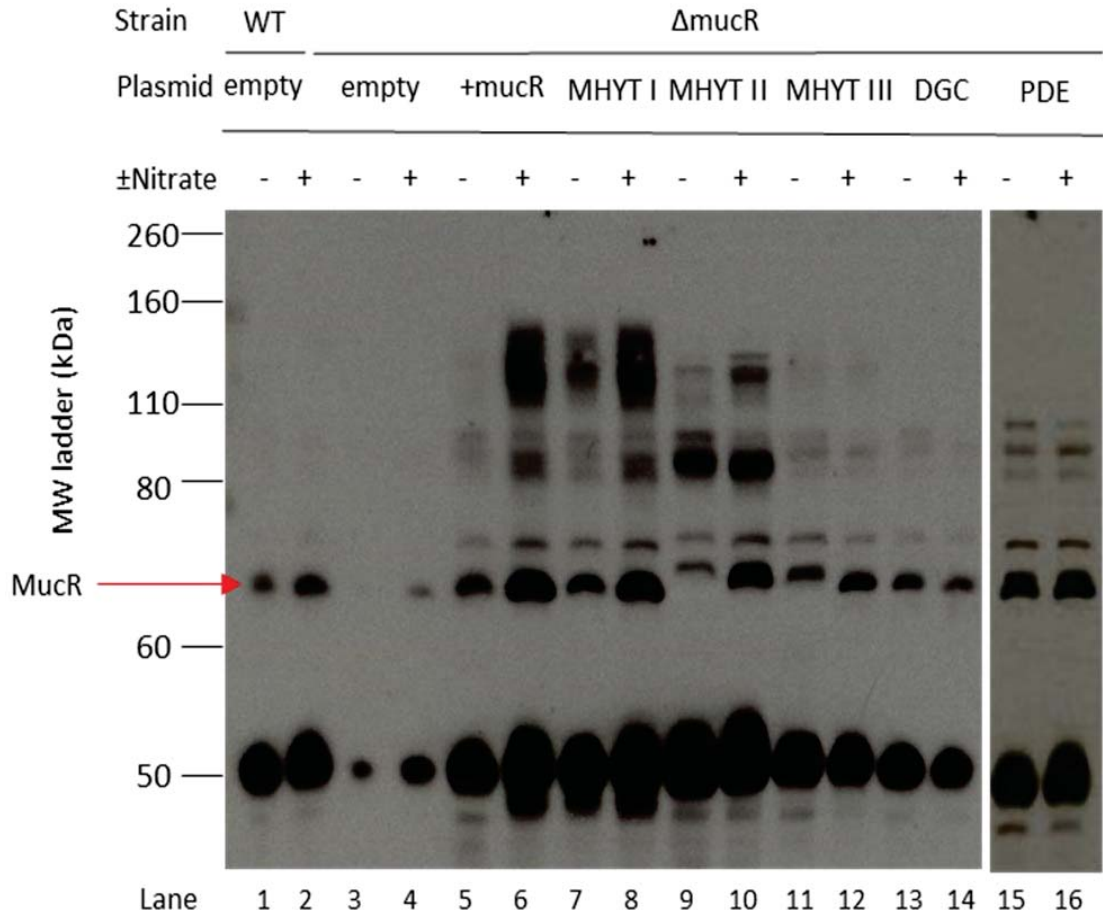


Figure 4.4 Immunoblot detection of MucR in inner membrane fractions of cell lysates.

Shown are immunoblots using anti-MucR antibody to detect MucR in the inner membrane fraction of the WT strain and *mucR* deletion mutant - harbouring various plasmids - grown in the absence (-) or presence (+) of nitrate during biofilm growth on solid medium. WT strain harbouring an empty vector (lanes 1-2). The *mucR* deletion mutant ($\Delta mucR$) expressing various plasmid-borne variants of *mucR* gene (lanes 3-16): empty vector (lane 3-4), normal *mucR* (lanes 5-6), *mucR* with defective sensor motifs (mutated MHYT I, MHYT II or MHYT III motifs) corresponding to lanes 7-8, 9-10 & 11-12), and MucR with defunct output domains (DGC or PDE, lanes 13-14 & 15-16). MW ladder shown in kDa. Red arrow = expected MW of MucR (74 kDa). Position of MW ladder bands are shown on the y-axis (Novex™ Sharp Pre-Stained Protein Standard, Invitrogen, USA). There is an anomaly in lane 4 - a faint band corresponding to the same MW as MucR detected in the sample from a *mucR* mutant (with an empty vector)

grown in the presence of nitrate. This unexpected band could be a background signal, a protein similar to MucR or accidental leakage from a neighbouring lane during loading.

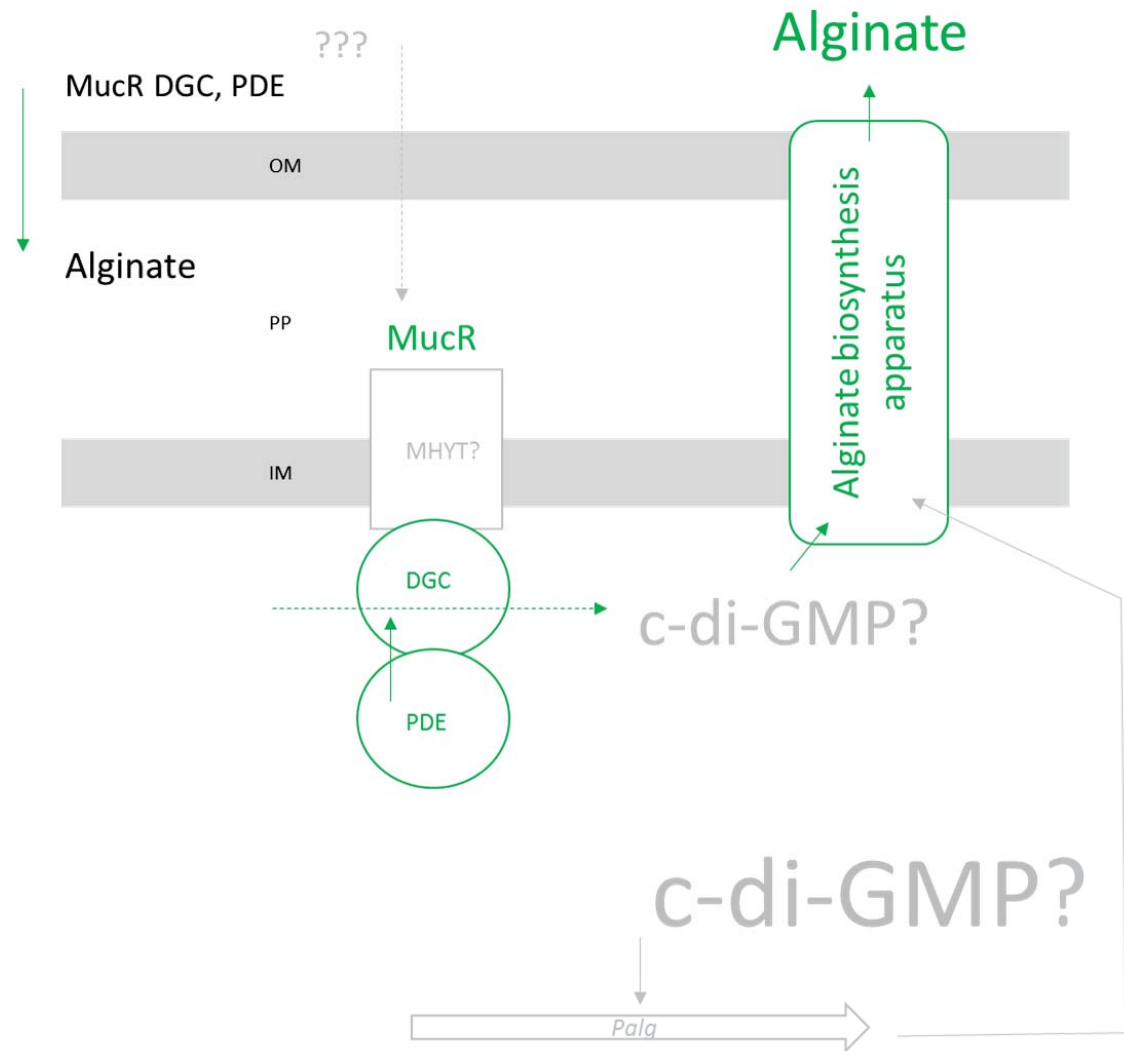


Figure 4.5 Section 4.1 results suggest that MucR's DGC & PDE domains are important for alginate production. Green parts are supported by experimental findings. Grey parts are hypothetical.

4.2 Nitrate impairs alginate production through MucR

In the previous section, it was shown that both c-di-GMP synthesising and degrading functions of MucR output domains were important for making alginate. However, so far it is still not known if these activities are affected by any signals detected by the putative sensor domain of MucR. The sensor domain is thought to harbour three MHYT sensor motifs for the perception of a diatomic gas such as oxygen, carbon monoxide and nitric oxide, which have been respectively linked to reduced alginate yields, impaired biofilm formation and enhanced biofilm dispersal (Bragonzi et al. 2005; de la Fuente-Nunez et al. 2013; Hassett 1996; Murray et al. 2012; Wood et al. 2007; Worlitzsch et al. 2002). It has been reported that *P. aeruginosa* can carry out denitrification under low oxygen and high nitrate conditions, releasing nitric oxide endogenously (Line et al. 2014; Williams et al. 1978). However, it is not clear whether MucR facilitates a suppression of alginate production under such conditions.

To investigate this, I grew the WT strain, its *mucR* deletion mutant (both harbouring empty vectors) and a *mucR* deletion strain expressing plasmid-borne *mucR* on plain PIA medium (containing gentamicin) or in PIA medium to which nitrate had been added as potassium nitrate at 1% w/v (~ 100 mM). The latter thus provided levels of the initial substrate for denitrification (i.e. nitrate) and at a concentration that maximizes cell growth (Hassett 1996). After three days of incubation, alginate yields were measured. If MucR mediated a negative effect of nitrate on alginate production, then adding nitrate to the growth medium would lower alginate yields in both the WT strain and the plasmid-borne expression strain but have no effect in the *mucR* deletion mutant. My findings supported the prediction about the role of MucR in mediating nitrate-induced suppression of alginate production (Figure 4.6, page 108), in that nitrate significantly

reduced alginate yields only in the strains containing an intact *mucR* gene (i.e. the WT and complemented strains).

To rule out the possibility that nitrate blocked alginate production by destabilizing MucR, leading to its degradation, I prepared inner membrane fractions and probed them with anti-MucR antibodies. If nitrate led to degradation of MucR, then the intensity of full length MucR might be reduced in immunoblots. However, my results show that nitrate did not abolish the presence of full length MucR (Figure 4.4, lanes 7-12, page 105).

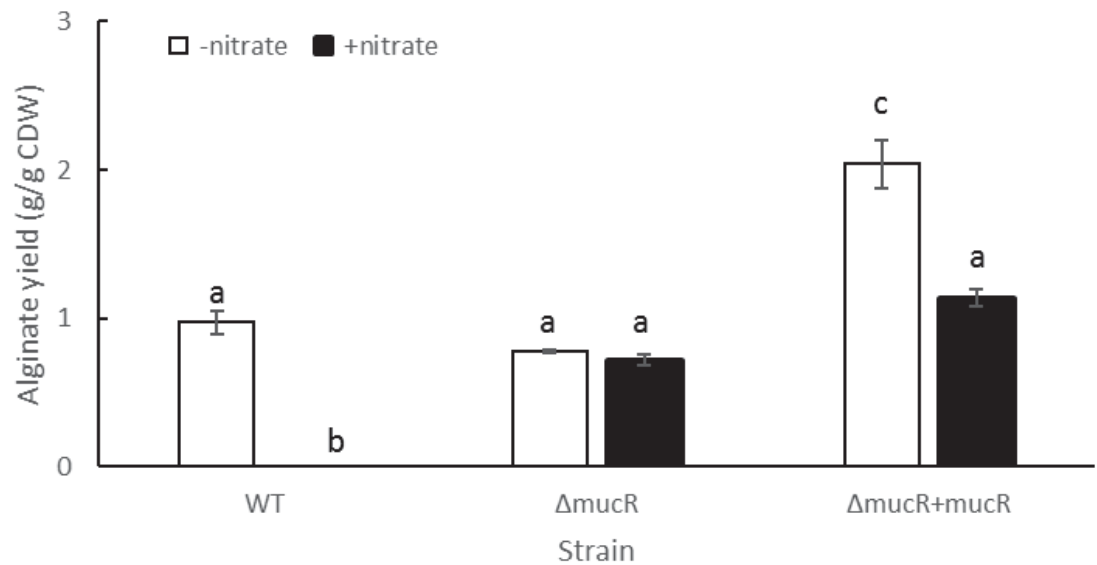


Figure 4.6 The role of MucR in nitrate perception and alginate production. Effect of nitrate and *mucR* on alginate yield. Shown are mean alginate yields \pm SE, representative of three independent experiments. Strains were grown in the absence (white) and presence (black) of nitrate. WT and Δ mucR = WT strain and *mucR* deletion mutant strains harbouring empty pBBR1MCS-5 vectors. Δ mucR+mucR = *mucR* deletion mutant expressing plasmid-borne *mucR*. Treatments with different letters are

significantly different (LSD Test, $n = 3$, $p < 0.05$). g/g CDW = grams of alginate per gram of cellular dry weight. Reused with permission from Springer Nature (Wang et al. 2015).

4.3 MucR's MHYT sensor domain is involved in nitrate perception

Because MucR played a role in nitrate suppressing alginate production (Section 4.2), I carried out experiments to determine whether MucR's sensor domain was involved in nitrate perception, leading to reduced alginate yields. Variants of MucR were generated with single, double, and triple histidine to alanine substitutions at their MHYT motifs (H59A, H122A, and H189A) - numbered I, II, and III from the N terminus. They were made by SLIM PCR in combination with gene synthesis, sequence verified and then cloned into pBBR1MCS-5 (Wang et al. 2015) (also *see* Section 2.3.4). I subsequently introduced variant-containing plasmids into *E. coli* S17-1 for transconjugation into the *mucR* deletion mutant (Section 2.3.11). Successful recipients were selected for on PIA medium containing gentamicin and received plasmids were confirmed by plasmid DNA isolation and restriction digestion (Figure 4.2B-C, page 101-102).

To investigate if the MHYT motifs of MucR's sensor domain were involved in nitrate perception, variants of MucR with individually mutated MHYT sensor motifs were expressed from pBBR1MCS-5 plasmids in the *mucR* deletion mutant. These strains were grown on PIA medium containing gentamicin with or without added nitrate. In parallel, the *mucR* deletion mutant expressing vector-borne unmutated *mucR* cultivated in the absence and presence of nitrate was included as control. After three days' incubation, alginate yields were measured. As expected, alginate yields of the control

strain were significantly reduced when nitrate was added to the growth medium (Figure 4.7, page 110), indicating that MucR is involved in nitrate perception.

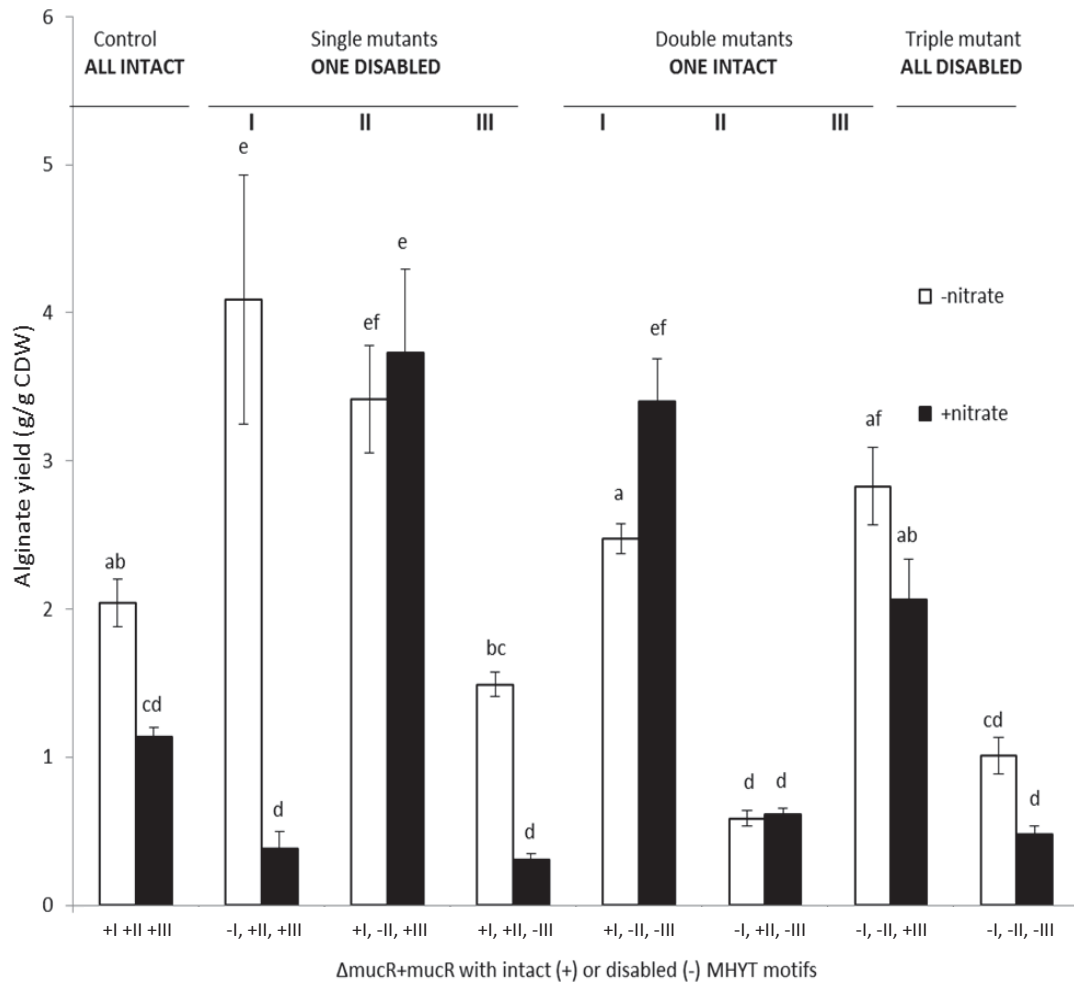


Figure 4.7 The role of MucR sensor motifs in nitrate perception and alginate production.

MucR has three MHYT motifs. Shown are the effects of nitrate and inactivation of MucR sensor motifs on alginate production ($n = 3$). Mean alginate yields \pm SE are shown, representative of three independent experiments. Strains were grown in the presence/absence (black/white) of nitrate. Here, in *mucR* mutant, variants of MucR were expressed from pBBR1MCS-5 plasmids. These variants have single, double and triple histidine to alanine substitutions at their MHYT sensor motifs (MHYT to MAYT mutation). g/g CDW = grams of

alginate per gram of cellular dry weight. Control = Δ mucR+mucR i.e. the *mucR* deletion mutant expressing plasmid-borne *mucR* with MHYT motifs **ALL INTACT** (+I, +II, +III). Single mutants have only **ONE DISABLED** motif, double mutants have **ONE INTACT** motif, and the triple mutant has its motifs **ALL DISABLED**. Notation on x-axis dictates which variant of *mucR* was expressed in the *mucR* deletion mutant with roman numeral dictating which MHYT motif was disabled (-) or left intact (+). Treatments with different letters are significantly different (LSD Test, n = 3, p < 0.05).

Disruption of MHYT I in the absence of added nitrate or disruption of MHYT II regardless of whether nitrate was added to the growth medium did not significantly reduce alginate production. Strains expressing MucR with either inactive MHYT I or MHYT III sensor motifs exhibited reduced alginate yields when grown in the presence of nitrate compared to respective strains grown in the absence of added nitrate (Figure 4.7, page 110). These results suggest that (1) MHYT I and III are apparently not necessary for perception of nitrate signal, (2) an intact MHYT II is involved in nitrate perception, and (3) MHYT III enhances alginate production. To rule out the possibility that the introduced mutations (or nitrate itself) impaired alginate biosynthesis by destabilizing MucR, leading to its degradation, I probed inner membrane fractions with anti-MucR antibodies (Section 2.6.7). My immunoblot experiments show that neither nitrate nor single histidine to alanine substitutions detrimentally affected MucR stability (Figure 4.4, lanes 7-12, page 105).

To consolidate above findings about the functions of MHYT motifs, I carried out further experiments on MucR with double and triple mutations. As shown in Figure 4.7 on page 110, alginate yields of the strain containing an intact MHYT I motif (with inactivated MHYT II & III motifs) did not respond negatively to nitrate. Alginate yields

of the strain with only an intact MHYT II motif (and disrupted MHYT I & III motifs) was reduced to background levels regardless of whether nitrate was added to the growth medium, and alginate production levels of the strain bearing the normal MHYT III motif (with mutated MHYT I & II motifs) also did not respond to nitrate. Furthermore, when all three motifs were knocked out from the sensor domain, alginate production was reduced to background levels regardless of whether nitrate was added (Figure 4.7). These findings support the conclusions derived from the single mutant experiments (Figure 4.7, page 110).

Based on the results from this section, I proposed a model to illustrate how the MHYT motifs and nitrate may affect alginate production (Figure 4.8, page 113). In this model MHYT I and MHYT III motifs enhance alginate production while MHYT II impairs alginate production, and the latter effect is enhanced in the presence of nitrate (Figure 4.8, page 113). In essence, a delicate balance between positive and negative effectors dictates the eventual alginate production levels.

How this delicate balance is manifested mechanistically remained to be investigated. In the later parts of this study, I attempted to explore potential mechanisms of nitrate-induced suppression of alginate production, for example, whether nitrate affected MucR output domain activity by influencing its oligomeric state (Section 4.4); whether MucR and nitrate impacted alginate yield by influencing alginate promoter activity (Section 4.5), and whether MucR and nitrate influenced alginate production and promoter activity by influencing intracellular c-di-GMP levels (Section 4.6).

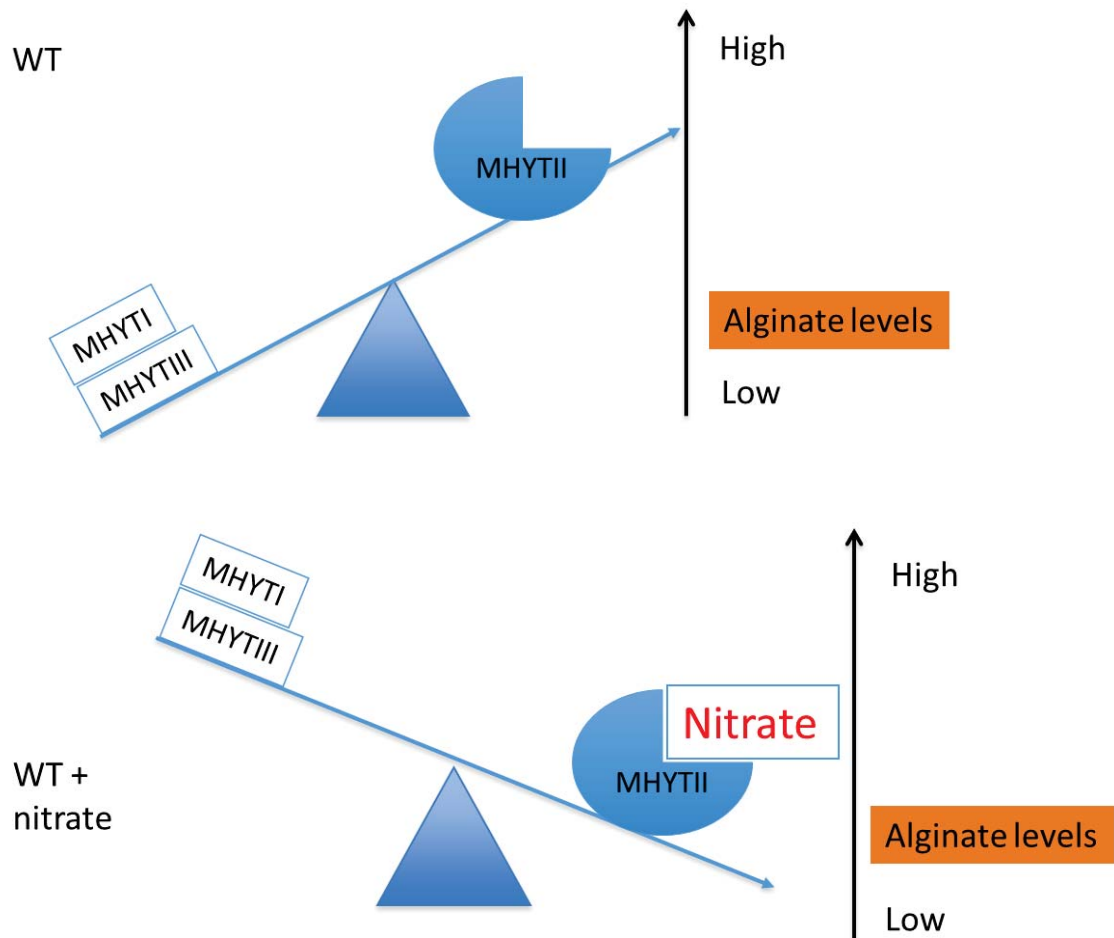


Figure 4.8 Schematic representation of how the MHYT motifs and nitrate affect alginate yield. A delicate balance between positive (MHYT I & III) and negative effectors (MHYT II & nitrate) dictates final alginate yield. MHYT I & III enhance alginate production while MHYTII impairs alginate production. Perception of nitrate by MHYTII amplifies this effect, suppressing alginate production. The large nitrate box represents growth conditions in the presence of added nitrate.

4.4 Effect of nitrate on MucR oligomeric state

In the previous sections, I showed that MucR perceived nitrate through its MHYT sensor domain, modulating alginate production levels. In principle, perception of nitrate may have altered the activity of MucR's output domain that is involved in c-di-GMP turnover. This could occur through various mechanisms. One possibility could be that nitrate affected the oligomeric state of MucR. To test if this was the case, I did crosslinking and western blot experiments. Here, the WT strain containing an empty vector was grown on PIA medium in the presence or absence of additional nitrate for three days. Biomass from plates was harvested and washed with either saline (for cells grown on normal PIA medium without additional nitrate) or saline supplemented with potassium nitrate (for cells grown in on PIA medium containing additional nitrate). The latter wash was used to help stabilise any interactions and/or oligomeric states that were nitrate sensitive.

Washed cells were incubated with or without a crosslinking reagent (DSG) as described in Section 2.6.4. The chosen reagent, DSG, was used to capture protein-protein interactions (i.e. potential oligomeric states of MucR). This water soluble homobifunctional NHS-ester-based crosslinker reacts with primary amines on the N-termini of peptides and the ϵ -amine of lysine residues, forming amide bonds between neighbouring (and within individual) proteins, stabilizing interactions that would otherwise be destroyed during the harsh conditions of sample preparation. Hence, these treatments would help stabilize multimeric complexes for immunoblot analysis with anti-MucR antibodies.

As negative controls, another set of cells grown under each condition was prepared and incubated without DSG. Subsequently, inner membrane fractions of DSG-treated and untreated cells were prepared (Section 2.6.2), quantified (Section 2.6.3), boiled in a

denaturing buffer and ran on SDS-PAGE (Section 2.6.6) prior to probing with anti-MucR antibodies (Section 2.6.7). If nitrate interfered with MucR activity by influencing its oligomeric state, then adding nitrate to the cultivation medium would also affect MucR oligomerization but this was not the case (Figure 4.9, page 116). Under both conditions, MucR appeared as a monomer (~75 kDa) in the non-crosslinked cells and a multimer (~300 kDa) in DSG-treated cells, suggesting that nitrate does not affect MucR oligomeric state, which appears to be multimeric by default. These new findings allowed me to revise the working model proposed earlier (Figure 4.5, page 106) into Figure 4.10 (page 117), which shows that nitrate does not modulate alginate production by altering the oligomeric state of MucR.

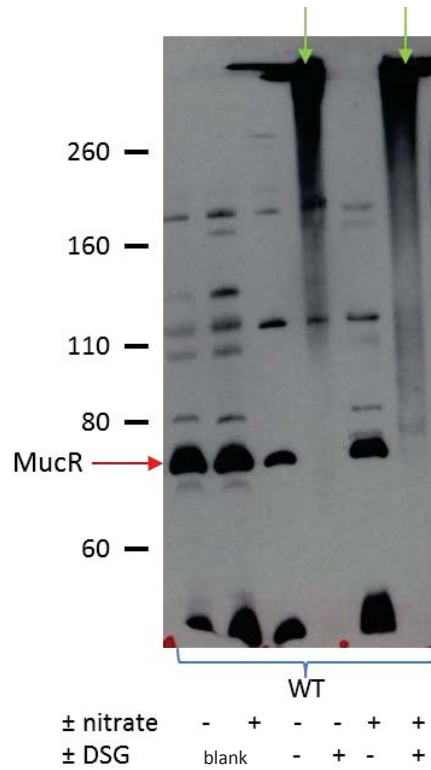


Figure 4.9 Crosslinking and immunoblot detection of MucR to examine effect of nitrate on MucR oligomeric state. From left, WT strain harbouring an empty vector was grown in the absence (-) or presence (+) of nitrate. Washed cells were either lysed directly (blank), or treated with (+) or without (-) DSG crosslinker inner membrane fractions were prepared and probed with anti-MucR antibodies. Left scale, MW ladder (kDa). Red arrow = expected MW of MucR. Green arrow = MucR in higher MW complexes. Position of MW ladder bands are shown on the y-axis (Novex TM Sharp Pre-Stained Protein Standard, Invitrogen, USA).

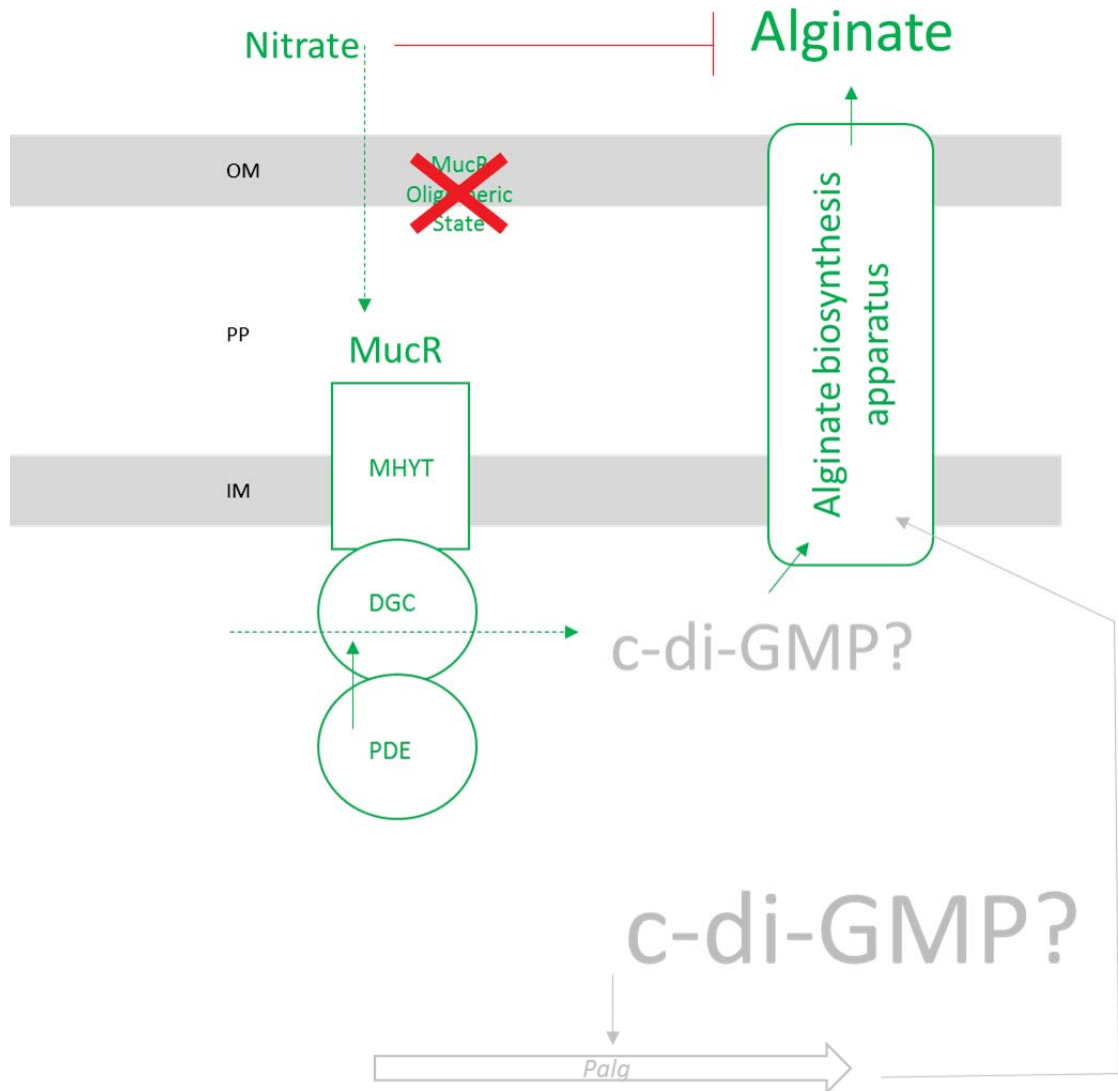


Figure 4.10 Summary of results from Section 4.1 to 4.4. I show that MucR's DGC & PDE domains are important for alginate production (Section 4.1), nitrate suppresses alginate production through MucR (Section 4.2), MucR's MHYT sensor domain is involved in nitrate perception (Section 4.3), and nitrate does not modulate alginate production by affecting the oligomeric state of MucR (Section 4.4).

4.5 Effect of MucR and nitrate on alginate promoter activity

In the preceding sections it was proposed that MucR was necessary for nitrate-induced suppression of alginate biosynthesis. To determine if this occurred at a gene expression level, I introduced an integration-proficient alginate-promoter-*lacZ*-reporter construct (miniCTX:*Palg-lacZ*) into the WT strain and its *mucR* deletion mutant, along with empty pBBR1MCS-5 vectors. This *Palg-lacZ* reporter construct contained a *lacZ* reporter gene controlled by the alginate promoter, a 1,000 bp region located directly upstream of the alginate biosynthesis gene cluster. Since the construct specifically integrates one copy of itself into the genome of recipient cells at the unique *attB* phage-attachment located at 6.24 Mb of the PAO1 genome (Ramos 2011), a comparison of alginate promoter activities between treatments would be possible by inference from LacZ reporter enzyme activity. Successful integration of the *Palg-lacZ* reporter construct into the genomes of recipient strains was confirmed by colony PCR (Figure 4.11A, page 119).

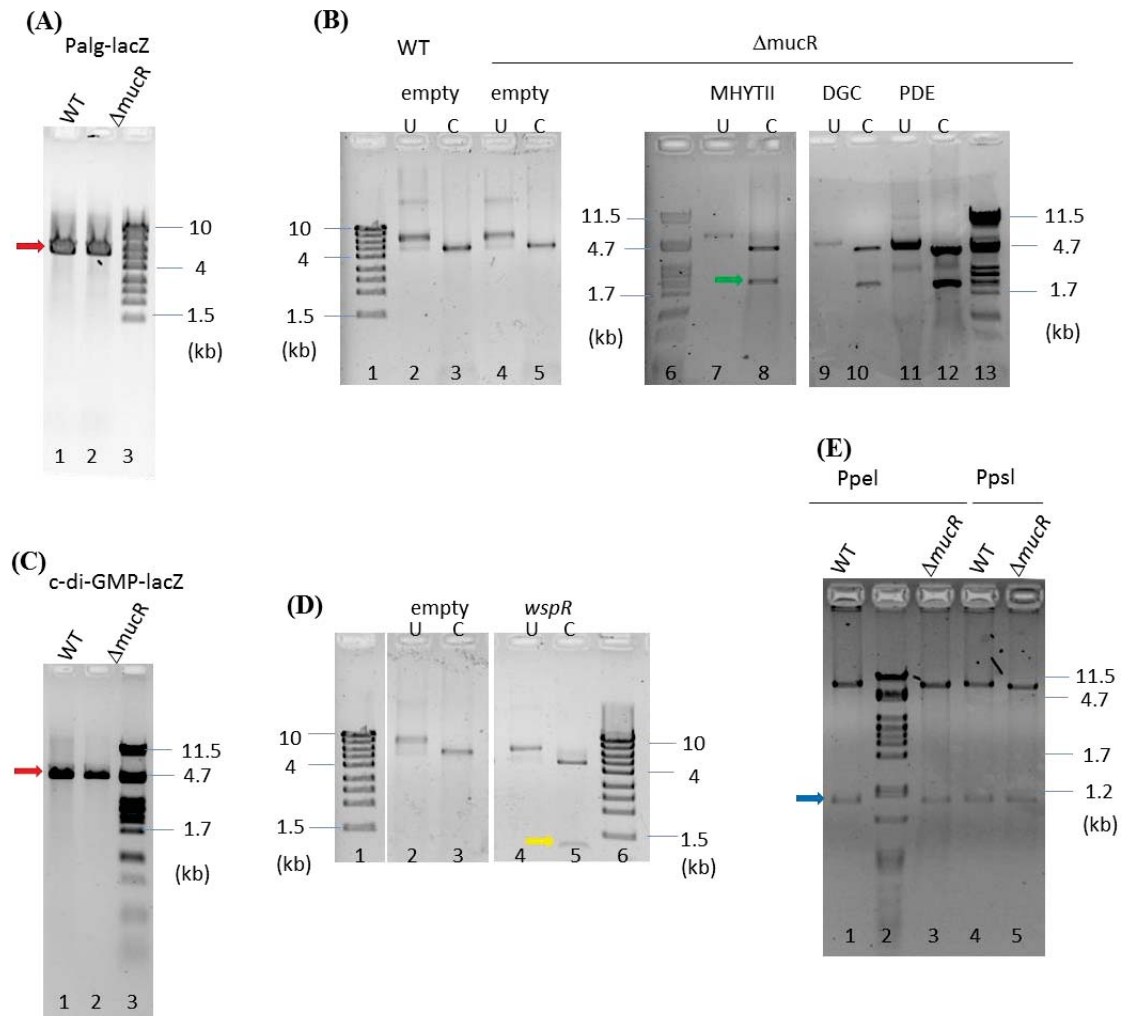


Figure 4.11 PCR confirmation of integrated *Palg-lacZ* and *c-di-GMP-sensitive lacZ* constructs and restriction confirmation of introduced plasmids. (A) Colony PCR confirming integration of *Palg-lacZ* reporter construct into genomes of WT strain and *mucR* deletion mutant. Red arrow = expected MW. **(B)** *ClaI/KpnI* restriction confirmation of pBBR1MCS-5 plasmids from strains harbouring genome-integrated *Palg-lacZ* constructs. Plasmids were empty or expressing variants of *MucR* with a defunct sensor (MHYT II) or output domains (DGC) or (PDE). Green arrow = *mucR* size. U = uncut, C = cut. **(C)** Colony PCR confirming integration of *c-di-GMP-sensitive-lacZ* reporter construct into genomes of WT strain and *mucR* mutant. Red arrow = expected MW. **(D)** *XbaI/SacI* confirmation of empty pBBR1MCS-5 vectors or vectors with *wspR* gene isolated from WT strain containing genomic

Palg-lacZ reporter. U = uncut, C = cut. Yellow arrow = *wspR* size. (E) BamHI/EcoRI verification of *Ppel* and *Ppsl* reporter plasmids (pTZ110*Ppel-lacZ* and pTZ110*Ppsl-lacZ*) introduced into strains. Blue arrow = size of *Ppel-lacZ* and *Ppsl-lacZ*. In panel (A), *left-panel* of (B), and panel (C) a MassRuler High Range DNA ladder (Fermentas, USA) was used. In *right-panel* of (B), and panels (C) & (E), a PstI-lambda DNA size marker was used.

To determine if nitrate-induced suppression of alginate biosynthesis occurred at a transcriptional level, the WT strain and its *mucR* deletion mutant – both harbouring *PalgD-lacZ* reporter constructs and empty PBBR1MCS-5 plasmids – were grown on PIA medium containing gentamicin, with or without additional nitrate. After three days' incubation, cells were harvested and *PalgD* activity was inferred from beta-galactosidase activity. If nitrate-induced suppression of alginate biosynthesis occurred at a transcriptional level, then alginate yields should somewhat correlate with alginate promoter (*PalgD*) activity, with a loss of alginate production in the WT strain (caused by nitrate) expected to be linked to reduced (or abolished) *PalgD* activity. Furthermore, deletion of *mucR* should not affect *PalgD* activity - since this mutant still produced normal alginate yields (independent of nitrate) (Figure 4.6, page 108).

However, my results show that while nitrate abolished alginate production in the WT strain (Figure 4.6, 108), it only reduced alginate promoter activity by 50% (Figure 4.12, page 121), suggesting that the negative effect of nitrate on alginate production was only partially caused by a drop in *PalgD* activity. Therefore, although nitrate suppressed alginate production through MucR, this was only partially dependent on alginate promoter activity. Hence, other forms of regulation are likely to be involved (Section 4.6). Since deletion of MucR also reduced alginate promoter activity (independent of

nitrate) (Figure 4.12, page 121), it could be important for expression of alginate biosynthesis genes.

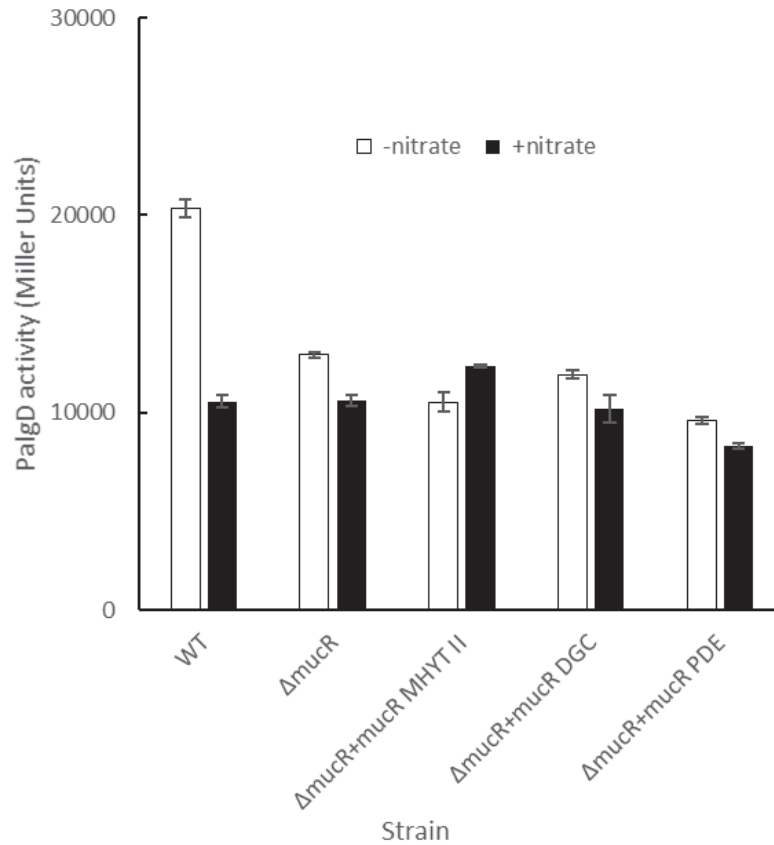


Figure 4.12 Effect of nitrate and MucR on alginate promoter activity. Shown are alginate promoter activities (Miller Units, mean \pm SE) of WT and *mucR* mutant strains grown in the absence/presence (white/black) of nitrate. *PalgD* activity was measured using a chromosomally integrated miniCTX*PalgD-lacZ* reporter. WT = PDO300 and Δ *mucR* = PDO300 Δ *mucR* strains harbouring empty pBBR1MCS-5 vectors. Δ *mucR*+*mucR* = *mucR* mutant harbouring variants of *mucR* with inactivated MHYT II, DGC or PDE domains. n = 3.

To determine if *PalgD* activity was dependent on MucR sensor and output domains, I also expressed variants of MucR with defunct sensor (MHYT II) (shown to impair nitrate perception) and output (DGC or PDE) domains in the mutant. These strains were confirmed by plasmid isolation and restriction digestion (Figure 4.11B, page 119). If the sensor and output domains of MucR were important for *PalgD* activity, then expressing defunct variants of MucR (with inactivated sensor or output domains) in the deletion mutant would not restore *PalgD* activity to WT levels. Indeed, none of these variants restored *PalgD* activity in the *mucR* deletion mutant (Figure 4.12, page 121), indicating that the MHYT II, DGC and PDE domains of MucR were all necessary for full alginate promoter activity.

My findings in this section show that alginate production yields were not entirely determined by alginate promoter activity and that when MucR was completely absent or when the above-mentioned domains were disabled, addition of nitrate did not affect alginate promoter activity. These results suggest that both the sensor and output domains of MucR are needed to elevate transcription of alginate genes beyond nitrate-repressed levels. To further validate this observation in future experiments it would be worth including as controls, measurements of *PalgD* activity for *P. aeruginosa* PAO1, a that does not produce alginate, and *PalgD* activity of a *mucR* mutant expressing non-mutated *mucR*.

4.6 Effect of MucR and nitrate on intracellular c-di-GMP levels

Results from the previous sections suggested that both MucR's sensor (MHYT) and output (DGC and PDE) domains were required for full alginate promoter activity but this activity did not directly determine final alginate yields. Therefore, other forms of regulation could be involved in nitrate induced suppression of alginate production. One possibility could be post-translational regulation. For instance, previous studies have shown that polymerisation of alginate requires binding of c-di-GMP to Alg44, activating the biosynthesis machinery at a post-translational level (Hay et al. 2009b; Moradali et al. 2017; Whitney et al. 2015). Moreover, this pathway could be driven by a local pool of c-di-GMP, presumably imparted by MucR (Hay et al. 2009b). However, to date, it has been technically challenging to directly measure local subcellular concentrations of this molecule.

An alternative approach could be used to obtain a proxy of local c-di-GMP concentrations by measuring the global pool through a non-invasive c-di-GMP-sensitive *lacZ* reporter gene (Section 2.9); non-invasive meaning that cells do not have to be lysed to assess c-di-GMP levels. Assuming that local pools governing alginate production substantially contribute to the global pool, this approach would give a relatively good idea of local c-di-GMP concentrations. Therefore, substantial changes to local concentrations – caused by nitrate or deletion of *mucR* – would also be detected as changes to global levels, as reflected by changes in LacZ reporter activity.

To this end, a c-di-GMP-sensitive *lacZ* reporter construct, mini-CTX-*lacZ*-*pelAwt* (Baraquet et al. 2012), composed of a *lacZ* reporter gene controlled by a promoter region (driven by a c-di-GMP-sensitive transcription factor, FleQ), was delivered into the WT strain and its *mucR* deletion mutant (Figure 4.11C, page 119). Since this construct integrates a single copy of itself into a specific location of the genome

(Baraquet et al. 2012), it should be possible to compare the relative global c-di-GMP levels between treatments of the same and different strains by inference from beta-galactosidase activity.

Resulting strains were cultivated on PIA medium containing gentamicin, with or without added nitrate. After three days' incubation, cells were harvested and beta galactosidase activity was measured. If nitrate blocked alginate production post-translationally by lowering local c-di-GMP levels, then adding nitrate to the growth medium of the WT strain would reduce global concentrations, as revealed by a drop in LacZ activity. My results support this prediction, i.e. the LacZ reporter indicated that nitrate significantly reduced global c-di-GMP levels in the WT strain (by 20%, Figure 4.13, 125), consistent with the possibility of a drop in local levels of this molecule and its role in reduced alginate production.

To examine if MucR was responsible for this drop, c-di-GMP levels of the *mucR* deletion mutant was also measured. If nitrate abolished alginate production through MucR by reducing local c-di-GMP levels, then deletion of *mucR* should block this effect. Indeed, when *mucR* was deleted, nitrate no longer significantly reduced c-di-GMP levels (Figure 4.13, page 125). This would support a role for a reduction of c-di-GMP levels in the nitrate-induced reduction of alginate synthesis, since, as described earlier (Section 4.2), this reduction also failed to occur when *mucR* was deleted. Deletion of *mucR* reduced global c-di-GMP levels by 40%, as inferred from beta-galactosidase activity (Figure 4.13, page 125) (twice as much as the effect of nitrate in the WT strain), suggesting that MucR contributes to both local and global concentrations of this molecule in a nitrate-dependent manner.

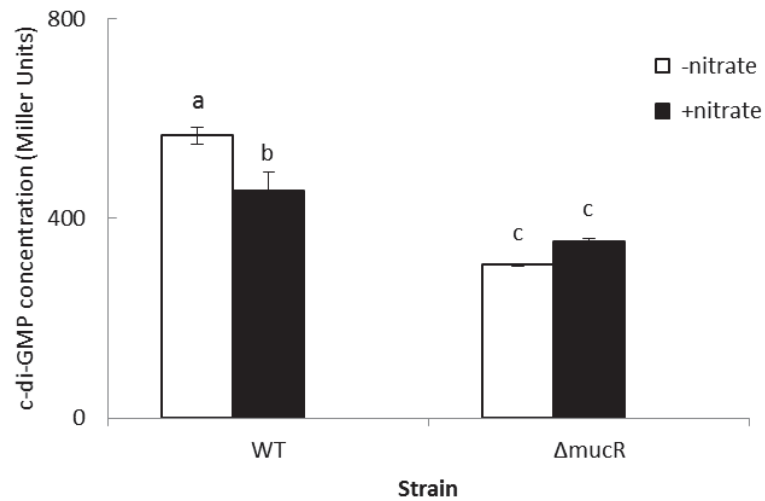


Figure 4.13 Effect of MucR and nitrate on global c-di-GMP levels. Relative global c-di-GMP levels (Miller Units, mean \pm SE) of strains grown in the absence/presence (white/black) of nitrate as inferred from beta-galactosidase activity of strains harbouring a c-di-GMP sensitive *lacZ* reporter construct . WT and Δ mucR = WT strain and *mucR* deletion mutant containing c-di-GMP sensitive *lacZ* reporter and empty pBBR1MCS-5 vector. n = 6, treatments with different letters were significantly different (LSD test, p < 0.05). Reused with permission from Springer Nature (Wang et al. 2015).

4.7 Effect of nitrate and intracellular c-di-GMP levels on alginate promoter activity

Results from the above sections suggested that nitrate suppressed alginate production through MucR. However, this was only partially linked to drops in c-di-GMP levels and *PalgD* activity, suggesting that neither of these exclusively determines alginate yields. Comparison of alginate yield, *PalgD* activity and c-di-GMP levels, revealed that the last two appeared to be better-correlated with each other than with alginate yields, leading to a prediction that *PalgD* activity could be driven by intracellular c-di-GMP levels. If so, then artificially increasing the global pool by over-expressing a highly active DGC would result in elevated *PalgD* activity. To test this hypothesis, I introduced a highly active DGC on a plasmid (pBBR1MCS-5:*wpsR*) into the WT strain harbouring the *PalgD-lacZ* reporter gene construct. This strain was cultivated on PIA medium containing gentamicin, with or without added nitrate, along with the same parent strain harbouring an empty vector as a negative control (these strains were confirmed by plasmid isolation and restriction digestion) (Figure 4.11D, lanes 1-4, page 119). I reasoned that if *PalgD* activity was sensitive to the intracellular c-di-GMP levels, then raising them would also increase *LacZ* reporter activity. Indeed, elevating c-di-GMP levels by over-producing *WspR* substantially increased *PalgD-lacZ* activity (Figure 4.14, page 127), suggesting that global c-di-GMP levels and *PalgD* activity were positively correlated. The conclusions regarding the role of MucR in alginate production resulting from all the above experiments are summarized in Figure 4.15 (page 128).

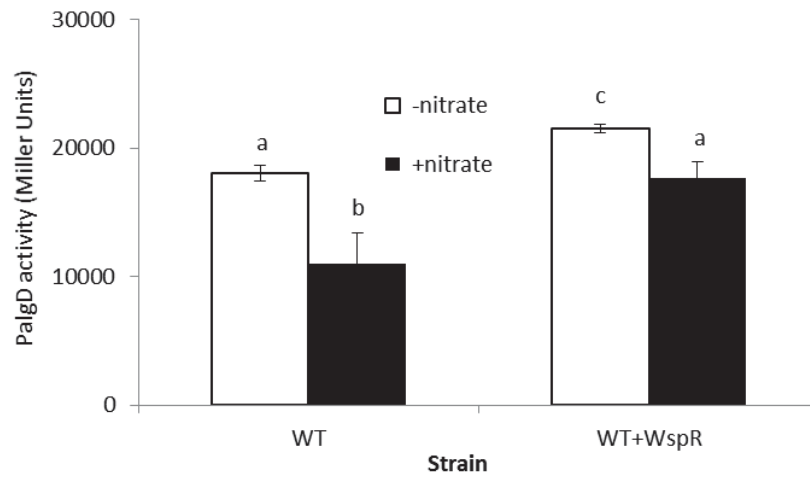


Figure 4.14 Effect of elevating intracellular c-di-GMP levels on alginate promoter activity.

Shown are alginate promoter activities (Miller Units, mean \pm SE) of WT strains grown in the absence/presence (white/black) of nitrate. *PalgD* activity was inferred from beta-galactosidase (*LacZ*) activity using a chromosomally integrated *PalgD-lacZ* reporters. WT and WT+wspR = WT strain (harbouring *PalgD-lacZ* reporter) with an empty vector or plasmid expressing a DGC, *WspR*, to artificially increase global c-di-GMP levels. n = 3, treatments with different letters are significantly different (LSD test, p < 0.05).

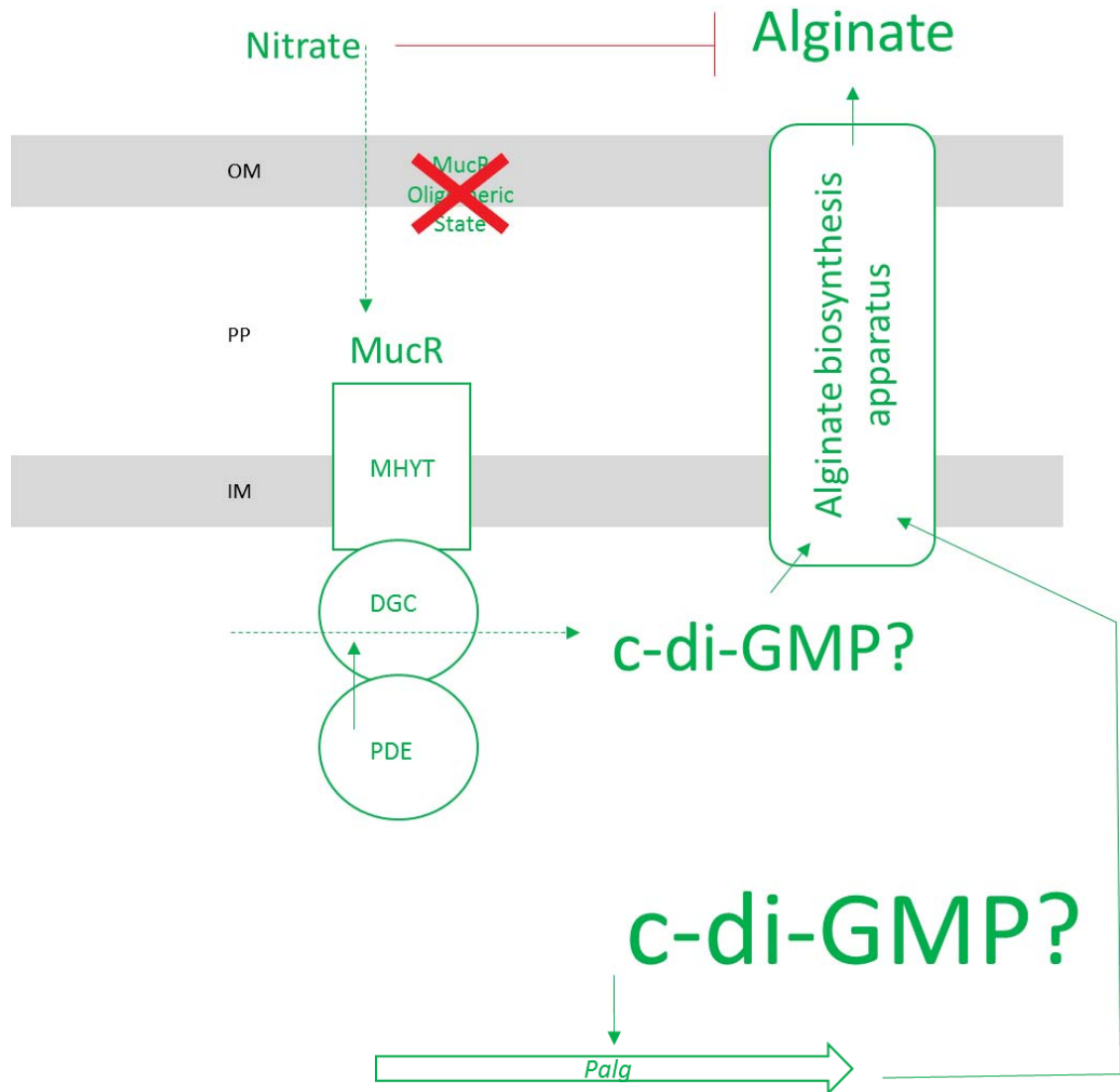


Figure 4.15 Results summarised for Sections 4.1 to 4.6. I show that MucR's DGC & PDE domains are important for alginate production (Section 4.1), nitrate suppresses alginate production through MucR (Section 4.2), MucR's MHYT sensor domain is involved in nitrate perception (Section 4.3), nitrate does not modulate alginate production by affecting the oligomeric state of MucR (Section 4.4), and in the WT strain, nitrate is sensed by MucR, leading to a slight reduction in c-di-GMP levels which then negatively affect alginate production (Section 4.5). It was also shown that nitrate, deletion of *mucR* or mutation of its sensor (MHYT II) and output (DGC or PDE) domains negatively affect alginate promoter activity (Section 4.6).

4.8 Effect of nitrate and MucR on other phenotypes sensitive to c-di-GMP

At high concentrations, c-di-GMP suppresses motility, favouring a sessile lifestyle, and in particular, the increase of c-di-GMP reduces swarming motility and enhances cell attachment, polysaccharide production and biofilm formation (Baker et al. 2016; Kuchma et al. 2015; Moradali et al. 2017; Zhu et al. 2016). Since addition of nitrate in the WT strain reduced c-di-GMP levels, as did deletion of *mucR* (Figure 4.13, page 125), I hypothesized that the MucR-nitrate pathway could also have impact on these other phenotypes.

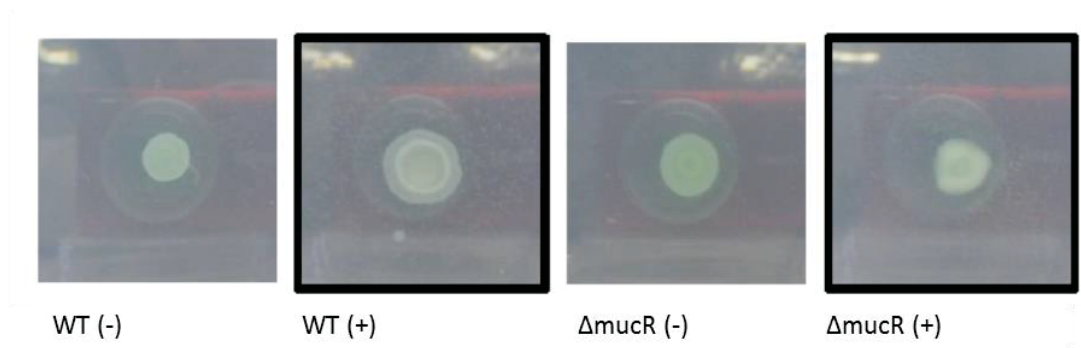
4.8.1 Swarming and attachment

To test the role of MucR and nitrate on swarming motility, the WT strain and its *mucR* deletion mutant harbouring empty vectors were subject to a swarm plate assay (Section 2.7). The results did not support such roles (Figure 4.16A, page 130), as comparable swarming (i.e. lack of) occurred in both WT and *mucR* mutant, regardless of nitrate addition.

Attachment, on the other hand, was reduced in the WT when nitrate was added, and when *mucR* was deleted (Figure 4.16B, page 130), to a very similar extent as the activity of the c-di-GMP-sensitive reporter (Figure 4.13, page 125). This suggests the

nitrate-MucR pathway influences attachment by impacting global c-di-GMP levels.

(A)



(B)

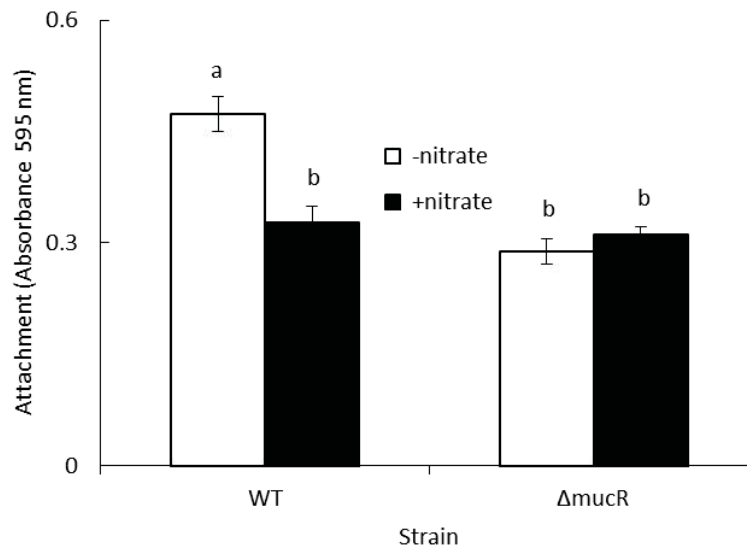


Figure 4.16 Effect of MucR and nitrate on swarming motility and surface attachment. (A)

Shown are swarming motilities of strains inoculated on swarming media supplemented with (+) or without (-) nitrate. Photographs are representatives of three independent experiments.

PDO300 = PDO300(pBBR1MCS-5) and ΔmucR = PDO300ΔmucR(pBBR1MCS-5). **(B)**

Attachment efficiencies (absorbance at 595 nm, mean ± SE) of strains upon exposure to the absence (white) or presence (black) of nitrate. Treatments with different letters are significantly different (LSD test, n = 7, p < 0.05).

4.8.2 Expression of Psl and Pel biosynthesis genes

The initial stage of biofilm formation, cellular attachment, is dependent on cellular appendages and secretion of exopolysaccharides (Colvin et al. 2012; Ma et al. 2009; Mikkelsen et al. 2011; Orgad et al. 2011; Wei and Ma 2013; Zhao et al. 2013). Aside from alginate, *P. aeruginosa* also produces at least two other exopolysaccharides, Psl and Pel (Colvin et al. 2012; Zhao et al. 2013). Previous studies showed that under conditions of elevated c-di-GMP levels, production of these polymers is substantially increased. Since the MucR-nitrate pathway also influences c-di-GMP levels, I hypothesised that it could also affect expression of Psl and Pel biosynthesis genes. To test this, I introduced plasmid-borne *Ppsl*- and *Ppel*-lacZ reporters, pTZ110:*Ppsl*-lacZ and pTZ110:*Ppel*-lacZ, into the WT strain and its *mucR* deletion mutant by conjugation. Successful transconjugants were confirmed by plasmid isolation and restriction confirmation (Figure 4.11E, page 119) and then grown on PIA medium containing carbenicillin in the presence or absence of additional nitrate, harvested, and relative promoter activities determined by measuring LacZ activity.

Using these reporters, I showed that while *psl* promoter activity was insensitive to nitrate, it was negatively influenced by *mucR* (Figure 4.17A, page 132). On the other hand, *pel* promoter activity was enhanced by nitrate, but only in the *mucR* deletion mutant (Figure 4.17B, page 132). These findings suggest that nitrate and MucR affect *psl* and *pel* promoter activities differently, possibly through mechanisms that are independent (or only partially dependent) on intracellular c-di-GMP levels.

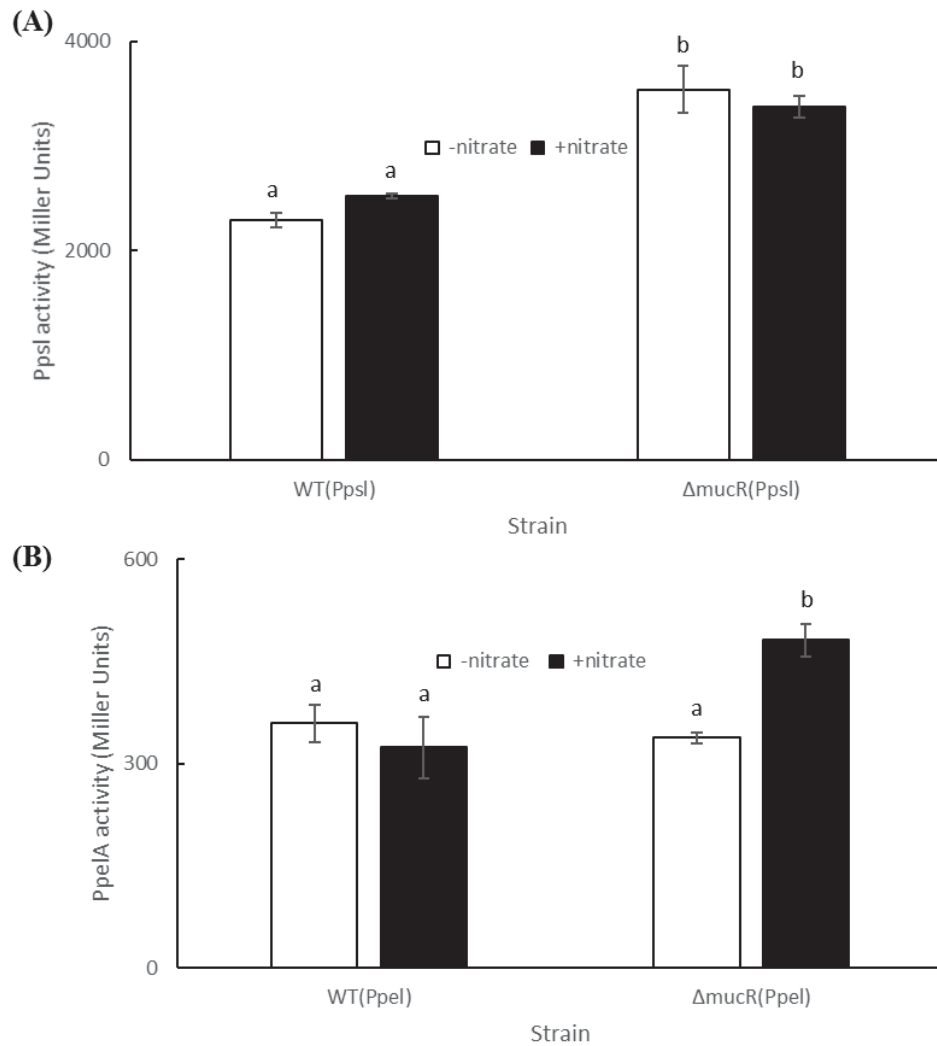


Figure 4.17 Effect of MucR and nitrate in transcriptional regulation of Psl and Pel polysaccharide biosynthesis operons. Promoter activities (Miller Units, mean \pm SE) of (A) *pslA* and (B) *pelA*, measured in PDO300 and Δ *mucR* using plasmid borne promoter-specific lacZ fusion reporters. White/black = grown in the absence/presence of nitrate. Treatments with different letters are significantly different (LSD Test, $n = 3$, $p < 0.05$). Reused with permission from Springer Nature (Wang et al. 2015).

4.8.3 Effect of nitrate and MucR on biofilm characteristics

The previous sections suggested that the MucR-nitrate pathway affected c-di-GMP levels, attachment efficiency and expression of genes involved in Psl and Pel production. Since these phenotypes are important for biofilm formation, for instance, Psl and Pel are involved in cell-surface and cell-cell interactions, it was hypothesized that the MucR-nitrate pathway might influence the biofilm lifestyle of *P. aeruginosa*. To see if this was the case, I cultured the WT and *mucR* mutant strains harbouring empty pBB1MCS-5 vectors on flow cell chambers (Section 2.8.2) in liquid PIA medium with or without added nitrate. After 24 h of cultivation, biofilms were stained (with a live/dead stain) and viewed by CLSM and analysed by IMARIS software (Section 2.8.3) to determine the biofilm thickness (height), cellular density, and ratio of live to dead cells.

My preliminary results show that both addition of nitrate and deletion of *mucR* led to formation of biofilms with greater thickness, compactness and survival (Table 4.1, page 134), supporting the possibility that the MucR-nitrate pathway influences biofilm lifestyle of *P. aeruginosa*. Interestingly, conditions that increased these biofilm parameters were also somewhat associated with increases in Psl and Pel promoter activity.

TABLE 4.1 Effect of MucR and nitrate on biofilm characteristics

	Nitrate	Average height	Average compactness	Ratio dead/alive
Strain	(±1% w/v)	vol (μm^3)/area (m^2)	Total flu./vol (μm^3)	Red/green flu.
WT	–	4.43	167	0.996
	+	6.71	379	0.307
ΔmucR	–	3.07	356	0.494
	+	8.17	528	0.267

flu. = fluorescence. vol. = volume. This experiment was performed once, hence can only be considered as preliminary. Reused with permission from Springer Nature (Wang et al. 2015).

4.9 Effect of MucR and nitrate on alginate production during planktonic growth

A recent study suggested that the function of MucR could be growth mode dependent (Li et al. 2013). To see if this was the case for nitrate sensing, the WT strain and its *mucR* deletion mutant (both harbouring empty pBBR1MCS-5 vectors) were grown in a chemically defined liquid medium designed to induce alginate production (Sections 2.5.2 and 2.5.3). Supernatants were harvested and uronic acid (UA) content (alginate yield) of unfiltered and filtered supernatants was determined. The quantity of UA in unfiltered supernatants represented the total alginate yield (i.e. high and low MW alginate) while UA of filtered supernatants represented the proportion of poorly made and/or degraded alginate (i.e. low MW alginate).

My findings show that in the absence of added nitrate, the WT strain produced 627 ± 19.6 and 106 ± 13.7 $\mu\text{g/ml}$ of alginate in unfiltered and filtered supernatants (Table 4.2, page 136). These values are significantly different (Student t-test, $n = 3$, $p < 0.05$),

indicating that the majority (~83%) of alginate produced by the WT strain in the absence of added nitrate was high molecular weight. However, when grown in the presence of added nitrate, the WT strain produced significantly less alginate in both fractions of the supernatant (i.e. $111 \pm 16.2 \mu\text{g/ml}$ in unfiltered and $101.3 \pm 13.1 \mu\text{g/ml}$ in filtered supernatants). Thus, cultivation of the WT strain in liquid medium with added nitrate led to production of small quantities of alginate that was essentially not made properly and/or substantially degraded. Similarly, knocking out *mucR* led to production of low quantities ($<120 \mu\text{g ml}^{-1}$) of poorly-made and/or degraded alginate in culture supernatants, regardless of whether nitrate was added to the growth medium (Table 4.2, page 136). Taken together, these findings suggest that during planktonic mode MucR is not only required for alginate production but it also mediates nitrate-induced suppression of alginate biosynthesis.

TABLE 4.2 Effect of MucR and nitrate on alginate (uronic acid) yields in unfiltered and filtered supernatants of strains grown in liquid medium

Strain	± Nitrate	UA of Unfiltered Supernatant (HMW+LMW alginate)	UA of Filtered Supernatant (LMW alginate)	% of high MW	*p-value
WT	-	627 ± 19.6	106 ± 13.7	~83	0.0001
WT	+	111 ± 16.2	101.3 ± 13.1	0	0.49
ΔmucR	-	42.2 ± 16.4	42.2 ± 23.1	0	1
ΔmucR	+	120 ± 19.0	109.1 ± 19.0	0	0.118

Shown are mean alginate yields (μg/ml) ± standard errors of unfiltered and filtered supernatants for the WT strain and its *mucR* deletion mutant, grown in the absence and presence of added nitrate. WT and ΔmucR = WT and *mucR* mutant harbouring empty pBBR1MCS-5 vectors. HMW and LMW = high and low molecular weight, respectively. UA = uronic acid. % of high MW alginate was calculated as follows: (UA of Unfiltered Supernatant – UA of Filtered Supernatant) / (UA of Unfiltered Supernatant x 100).

***Student t-test, n = 3, between alginate (uronic acid) yields of Unfiltered and Filtered supernatants for each treatment.**

5. Chapter Five: Discussion and conclusions

In this chapter, I attempt to interpret my major observations for the two proteins, AlgL and MucR, from this study with special reference to examining why disparity between my current results and previous reports occurred. I then summarise new and key information generated in my study and discuss the potential implications in manufacturing tailor-made bacterial alginate and management of *P. aeruginosa* biofilms.

5.1 Function of the alginate degrading enzyme AlgL

Encoded among the alginate biosynthesis genes is an alginate degrading enzyme, AlgL. Although it has been studied by several groups, inconsistencies have made it difficult to pin-point its role. Understanding the roles AlgL has in controlling alginate yield, polymer length and composition may inform future strategies to produce tailor-made alginate. Furthermore, if AlgL is essential for alginate production and/or biofilm formation, then it could be a potential target for managing *P. aeruginosa* biofilms.

In Chapter 3, I presented the results from studies that (1) investigated the function of AlgL and its lyase activity in alginate production, polymer length control and composition, (2) explored if AlgL was a free periplasmic protein or a subunit of the biosynthesis complex, and (3) examined its role in biofilm attachment and dispersal.

My findings suggest that the lyase activity of AlgL negatively influences alginate yield on solid medium, supporting previous work that plasmid-borne expression of alginate lyases detrimentally affect alginate production (Tatnell et al. 1996; Wang et al. 1987). My preliminary results also reveal that AlgL is dispensable for alginate biosynthesis during biofilm growth, which is aligned with previous findings from *P. syringae* and *P. aeruginosa* 8830 (Boyd et al. 1993; PenalozaVazquez et al. 1997) but contrary to those

reported for *P. aeruginosa* FRD1 (Albrecht and Schiller 2005; Jain and Ohman 2005; Monday and Schiller 1996). Recently it was suggested that the function of AlgL could be growth mode dependent. It was proposed that AlgL is required for alginate production during planktonic mode (Wang et al. 2016a). This finding is consistent with several earlier reports which suggested that deletion of *algL* in various alginate producing bacteria (*P. aeruginosa* FRD1 and PDO300, *P. fluorescens* and *A. vinelandii*) reduced alginate yields and/or interfered with polymer length control and epimerization degree during planktonic growth (Albrecht and Schiller 2005; Bakkevig et al. 2005; Jain and Ohman 2005; Trujillo-Roldan et al. 2003; Wang 2016). Therefore, the requirement of AlgL in alginate production could be species (strain) and/or growth mode dependent.

A species (strain) and/or growth mode dependent effect is exemplified by observations that AlgL is completely dispensable for alginate production in *A. vinelandii* (Trujillo-Roldan et al. 2003), dispensable only during biofilm mode in *P. syringae* (PenalozaVazquez et al. 1997) and *P. aeruginosa* PDO300 (preliminary findings presented here), or completely indispensable during both growth modes in *P. fluorescens* (Bakkevig et al. 2005) and *P. aeruginosa* FRD1 (Albrecht and Schiller 2005; Jain and Ohman 2005). One explanation for these disparities is that in species (strains) and/or growth modes where AlgL could be dispensable, the bacterium produces an alternative alginate lyase that is functionally similar to AlgL. This is plausible for *A. vinelandii* as it produces a bi-functional extracellular enzyme with both epimerization and beta-elimination activities (Svanem et al. 2001). By extension, other bacteria that can make alginate in the absence of AlgL could in principle also produce other lyases. This is possible since numerous alginate lyases (of different size, specificity or activity to AlgL) have been discovered in environmental *Pseudomonas* (Kraiwanapong et al. 1997; Lee et al. 2009; Lundqvist et al. 2012; Ott et al. 2001;

Wang et al. 2017; Zhu et al. 2015) and *Azotobacter* isolates (Davidson et al. 1977). However, it is still unclear whether such enzymes are also produced by *Pseudomonas* strains used in previous or in the present study. Hence, further experiments would be required to validate this explanation.

Alternatively, the requirement for AlgL could be an exclusively growth mode dependent phenomenon. Planktonic cells have low c-di-GMP levels which inherently suppress alginate production (Romling et al. 2013). Keeping alginate production switched off during planktonic mode would avoid the metabolic costs of producing a high molecular weight polymer that would compete with the energy demands of cell division, reduce oxygen and nutrient availability (by increasing supernatant viscosity) and innate protection against antimicrobial agents (which are comparably limited to biofilm cells). Therefore, making alginate during planktonic mode may not be physiologically necessary, advantageous or even permissible. This is supported by the observation that forcing planktonic cells to produce alginate in the absence of AlgL detrimentally affects alginate biosynthesis and cell growth (Albrecht and Schiller 2005; Bakkevig et al. 2005; Jain and Ohman 2005). As a result, it is possible that alginate production during planktonic mode is error-prone, hence requiring an alginate lyase to perform a maintenance role to clear the periplasm of misguided alginate. Conversely, during biofilm growth AlgL activity could be dispensable because translocation/secretion may be more reliable during this growth mode, or possibly other lyases are also expressed. However, additional experimental work would be needed to substantiate these explanations.

The third possible reason for why AlgL appears to be required for alginate production in certain species (strains) of bacteria may come down to differences in experimental setup (e.g., preparation of parent strains and generation of mutants). As outlined earlier on,

several different parent strains were used in previous studies, many of which were modified from ‘original’ environmental or clinical isolates to stabilize their alginate producing phenotype. For instance, stable mucoidity was maintained by introducing a native plasmid - encoding unknown genetic elements into *P. syringae* (PenezolaVazquez et al. 1997), or by chemical mutagenesis in *P. fluorescens* Pf201 and *P. aeruginosa* 8830 – which would have also introduced unknown mutations (Bakkevig et al. 2005; Boyd et al. 1993; Darzin et al. 1984). To avoid such effects, several research groups placed the *algC* gene (in *P. fluroescens* Pf201) or alginate operon (in *P. aeruginosa* FRD1) under the control of inducible promoters prior to disrupting the *algL* gene (Albercht and Schiller 2005; Bakkevig et al. 2005; Jain & Ohman 2005). While this approach offered excellent control over alginate biosynthesis, it completely disconnected the system from native transcriptional control. In contrast, the present study attempted to use the natural ‘switch’ of *P. aeruginosa* to stabilise mucoidity, which may produce more reliable results. However, further studies are required to pinpoint how preparation of parent strains affects the functions of *algL* and other genes.

The present study endeavoured to make a non-polar isogenic marker-free *algL* mutant with the intention to side-step potential technical challenges faced by other groups using transposon or marker-based strategies (Albercht & Schiller 2005; Boyd et al. 1993; Jain & Ohman 2005; Monday & Schiller 1996, PenazolaVazquez et al. 1997; Trujillo-Roldan et al. 2003). Although the complementation method used in this study recovered alginate yields, it failed to restore other phenotypes (e.g., O-acetylation degree and biofilm formation), alluding to potential polar effects on downstream genes. Moreover, since re-introducing *algL* on a medium-copy-number plasmid under a strong inducible promoter appeared to lower average polymer length and widened its size distribution, suggests that the complemented strain might be producing too much AlgL, negatively

affecting polymer length control, O-acetylation degree and biofilm formation. Thus a different complementation approach, perhaps introducing a single copy of the *algL* gene into the genome under its native promoter could be explored in future work.

Furthermore, the differences in growth conditions and methods for alginate yield measurement between studies may also contribute to the above mentioned disparities. For example, strains were often grown in (or on) different types of media - supplemented with carbon sources and/or compounds to further enhance/stabilise alginate production. However, it is difficult to tell whether these variations have affected findings reported thus far. Therefore, it might be worth comparing alginate yields of species (strains) grown under the same conditions. It is also noted that various studies have used different methods for measuring alginate yields, for example, mucoidy on solid medium (Boyd et al. 1993), a carbazole assay for strains grown in liquid or on solid medium (Albercht & Schiller 2005, Monday & Schiller 1996, Jain & Ohman 2005; PenazolaVazquez et al. 1997) and an end-point alginate lyase assay (Bakkevig et al. 2005). Although all these methods are reasonably dependable, they each have shortcomings. For instance, a mucoid phenotype does not show relative changes in alginate yields, the carbazole assay is susceptible to interference with neutral sugars and the end-point assay is also affected by other compounds absorbing at the same wavelength. Therefore, to make findings comparable, the same measuring methods should be used. Moreover, the method used to quantify alginate yields in the present study offers greater specificity for uronic acids (Blumenkrantz and Asboe-Hansen 1973). Although one cannot completely ignore the possibility that *Pseudomonas* spp. could also produce other uronic acid containing compounds in the biofilm matrix.

Although the requirement of AlgL in alginate production could potentially be a species/strain and/or growth mode dependent phenomenon, additional work would be

required to validate this possibility. Moreover, due to differences in experimental design, methodology and technical challenges encountered in previous and current work it is difficult to completely explain the discrepancies observed. Thus, more studies would be required to fully appreciate the function of AlgL in alginate biosynthesis and regulation.

While previous models propose that AlgL serves a maintenance role in the periplasm, they disagree on whether it is a structural component of the complex or a free periplasmic protein. Given its co-localization and (presumed) co-expression with other alginate biosynthesis subunits and the fact that catalytically inactive AlgL can enhance alginate yield during biofilm growth (Section 3.1), it is possible that AlgL is a structural component involved in translocation/secretion, as originally proposed by Jain & Ohman (2005). Indeed, AlgL might also be a structural component of the complex during planktonic mode since deletion of *algL* during this growth mode impaired alginate production, molecular mass and epimerisation degree in several different strains of alginate producing bacteria (Albrecht and Schiller 2005; Bakkevig et al. 2005; Jain and Ohman 2005; Trujillo-Roldan et al. 2003).

However, preliminary results of this study showed that the deletion of AlgL during biofilm growth did not impair the function (alginate yield, molecular weight or composition) or stability of the complex, suggesting that AlgL could be a free periplasmic protein. Furthermore, AlgL was not found to interact with several different subunits of the complex. However, given technical limitations of the interaction studies performed here, at this stage I cannot exclude the possibility that AlgL is interacting through weak/transient interactions that were difficult to detect, or with other subunits that were not included in this experiment. Therefore, future studies should include all relevant mutants and complemented strains. Additionally, using other tags and/or

tagging of other subunits could be explored because placing a highly positively charged hexa-histidine tag at the c-terminus of AlgL may interfere with its protein interactions. Moreover, a positive control should be included to ensure that the method used can effectively detect interactions already previously demonstrated in the literature.

Although the apparent effects of AlgL and its lyase activity on alginate yield, molecular weight and composition had no direct effect on biofilm attachment, growth or dispersal, my study suggests an association between O-acetylation degree and attachment. This finding supports previous claims that this modification enhances surface colonization, micro-colony formation and cell-cell interaction (Nivens et al. 2001; Tielen et al. 2005). In the present study, there was also a tentative effect of O-acetylation degree on biofilm dispersal. However, its significance would require further experiments to elucidate.

Alginate lyases have been shown to work in synergy with antimicrobial agents in eradicating mucoid *P. aeruginosa* biofilms (Alipour et al. 2009; Alkawash et al. 2006; Bayer et al. 1992; Islan et al. 2015; Smyth and Hurley 2010). Degradation of alginate by these enzymes not only enhances the penetration of antimicrobial agents into the biofilm, but also releases unsaturated alginate oligomers which are a potent stimulant of innate host immune responses including production of cytokines (TNF), nitric oxide and reactive oxygen species (Fang et al. 2017; Iwamoto et al. 2005; Xu et al. 2015; Xu et al. 2014). Since nitric oxide concentration is low in CF lungs (de Winter-de Groot and van der Ent 2005), release of alginate degradation products by lyase activity might enhance inflammatory responses to help get rid of invading bacteria. Thus, alginate lyases not only break down the biofilm, exposing bacterial cells to antimicrobial agents and phagocytosis, but could also enlist other host immune responses to help defeat the infection.

5.2 Function of the inner membrane protein MucR

As described in the introduction, the alginate biosynthesis machinery is activated post-translationally by c-di-GMP, imparted by the inner membrane protein MucR. However, it was unclear how this occurred. In Chapter 4, I attempted to elucidate how MucR regulated alginate production and biofilm formation to provide insights into ways of harnessing MucR function to manipulate alginate biosynthesis and biofilm development in favour of managing *P. aeruginosa* biofilms and production of tailor-made alginates. Specifically, I (1) studied the role of MucR's sensor and output domains in alginate production, (2) explored if nitrate was a putative signal perceived by MucR, (3) assessed whether MucR-nitrate pathway regulated alginate production through c-di-GMP at post-translational/transcriptional level(s), and (4) examined the broader implications of this pathway on biofilm lifestyle e.g. swarming, attachment, expression of Psl and Pel biosynthesis genes, and biofilm thickness, compactness and survival.

I demonstrate for the first time that MucR was required for alginate production during planktonic mode. However, my finding that MucR was dispensable for alginate production during biofilm growth is contrary to a previous report (Hay et al. 2009b). This disparity may be due to differences in experimental design. In both studies, identical strains were initially grown under the same conditions as pre-cultures. However, strains were prepared differently for inoculation on PIA plates. In particular, I included additional steps in the present study to minimise potential effects associated with supernatant components, inoculum size and medium quantity. Since the previous study did not control for these variables, results of the present study could be more reliable and accurate. In spite of these differences, both the current and previous studies (Hay et al. 2009b) show that over-expression of MucR increased alginate production, indicating that MucR has a positive effect on alginate yields during biofilm growth.

Here, my findings suggest that both DGC and PDE output active sites of MucR are important for alginate biosynthesis. This finding refines previous observations that a complete lack of the MucR output domain impaired alginate production (Hay et al. 2009b). While the majority of bi-modular (DGC + PDE) enzymes have only one active domain, both domains can be simultaneously active in some cases e.g., MSDG1 of *Mycobacterium* (Bharati et al. 2012; Sharma et al. 2014), alternately/conditionally active e.g. ScrC in *Vibrio parahaemolyticus* (Ferreira et al. 2008) or completely inactive e.g. FimX in *Xanthomonas citri* (Navarro et al. 2009).

My finding (and those of others) that the function of MucR was growth mode dependent suggests that the output domains of MucR could be conditionally active (Li et al. 2013). Such a mode of activation could be facilitated by protein-protein interactions or signal perception by sensor domains. For example, the activity of the bi-modular enzyme, ScrC of *Vibrio parahaemolyticus* (which is involved in the switch between swarming and biofilm forming states) is activated alternately/conditionally by protein-protein interactions (Ferreira et al. 2008). Specifically, its interaction with regulatory proteins ScrA and ScrB activates its PDE activity while in the absence of ScrA and ScrB, ScrC behaves as a DGC (Ferreira et al. 2008). Furthermore, blue light is a signal that activates the DGC activity of BphG1, a bacteriophytochrome of *Rhodobacter sphaeroides* (Tarutina et al. 2006). Here, it was shown that nitrate could be a signal that mediates the activity of MucR output domains (see below).

The sensor domain of MucR has MHYT motifs thought to perceive specific signals that modulate the activity of its output domain. In the present study, nitrate was identified as a potential signal that suppresses alginate production through MucR's second MHYT sensor motif. I show here that MHYT I & III positively affect alginate yields while MHYT II (and its perception of nitrate) negatively affect alginate yields, suggesting that

alginate production is dependent on a delicate balance between these positive and negative effectors. Although it is unknown how this delicate balance is manifested mechanistically, I made attempts to understand how the MucR-nitrate pathway detrimentally affected alginate yields. Initially, it was hypothesised that nitrate modulated the activity of MucR by affecting its oligomeric state. However, my results rejected this prediction. Because MucR was detected as a multimer by default independent of nitrate, it is conceivable that other proteins could be interacting with MucR and negatively affecting alginate yields.

The MucR-nitrate pathway might negatively affect alginate production transcriptionally by influencing intracellular c-di-GMP levels. However, my findings suggest that while both c-di-GMP levels and *PalgD* activity are somewhat correlated to each other, neither directly determines alginate yields. Thus, MucR-nitrate might impact alginate yields at multiple levels through pathways partially dependent on c-di-GMP levels. Multiple levels of regulation using different mechanisms might increase the robustness of cellular signalling.

Although the current work provides compelling evidence that nitrate impairs alginate production through MucR, elucidating whether it perceives nitrate directly or indirectly will require further experiments. Since *P. aeruginosa* can utilise nitrate as an alternative terminal electron acceptor during low oxygen conditions such as during biofilm growth (Cutruzzola and Frankenberg-Dinkel 2016), and denitrification intermediates have been linked to reduced alginate yields (Vollack and Zumft 2001; Wood et al. 2007; Worlitzsch et al. 2002; Zumft 1997), it is plausible that MucR may be detecting nitrate indirectly – possibly through denitrification intermediates and/or shifts in redox potential. To clarify this, future work could measure the emission of such intermediates to see if denitrification is indeed occurring. If it is, then cells could be grown in the

presence of specific intermediates to see if they lower alginate yields, and if they do, perhaps one could knockout specific genes in the denitrification pathway to see which denitrification product suppresses alginate production. Alternatively, other compounds that alter cellular redox potential in a similar manner to nitrate and/or its denitrification products could be tested to see if MucR elicits a more general effect on alginate yields in response cellular redox potentials.

Previous reports show certain intermediates (i.e. nitric oxide and nitrite) at physiologically tolerable concentrations – can be used to treat bacterial airway infections (Major et al. 2010; Yoon et al. 2006; Zemke et al. 2014). One of these intermediates, NO, is an important player of healthy innate host immune responses. However, in the CF lung, both NO and its carrier, S-nitrosoglutathione (GSNO), are virtually absent – potentially leaving patients susceptible to infection (Snyder et al. 2002). Thus, inhaling aerosols containing nitrate, denitrification intermediates and/or GSNO may help treat or prevent these infections by increasing the effectiveness of nitro-active host immune defences. Furthermore, since GSNO has been implicated in proper functioning of the lungs [by enhancing ciliary motility, relaxing airway smooth muscle and inhibiting airway epithelial amiloride-sensitive sodium transport (Snyder et al. 2002)], administration of these compounds along with biofilm degrading enzymes and/or antibacterial agents could be an effective recipe to manage CF lung infections. Indeed, there are patents for such products under development (Gaston and Stamler 2004).

It is worth noting that the PIA medium used in this study also contains background levels of nitrate (or denitrification intermediates), which MucR could be sensing. Therefore, one could replace the complex PIA medium with a predefined medium devoid of nitrate (subject to purity of ingredients supplied by manufacturers). This

would help determine if a complete lack of nitrate (or denitrification intermediates) would affect alginate yields. Alternatively, simulated mucosal media or CF lung infection models could be used to evaluate the physiological relevance of nitrate and denitrification intermediates on alginate production by *P. aeruginosa* (Yu et al. 2000). Moreover, since nitrate concentrations used in this study generally exceed normal levels encountered in lungs, future studies could use more physiologically relevant (or tolerated) concentrations to assess clinical applicability.

It has been suggested that elevations of c-di-GMP levels enhance attachment, polysaccharide production and biofilm development (Romling et al. 2013). Consistent with this understanding, my results indicate that the negative effects of MucR-nitrate interaction on c-di-GMP levels also reduced attachment efficiency. Interestingly, reduced c-di-GMP and attachment were not associated with reduced expression of promoters controlling the expression of Psl and Pel biosynthesis genes – two polysaccharides involved in cell-cell and cell-surface interactions. Instead, these promoters responded differentially to MucR and nitrate, potentially contributing to the already highly complex regulatory network controlling and fine-tuning the production of exopolysaccharides and other matrix components during biofilm development (Ghafoor et al. 2011).

Since Psl and Pel have somewhat similar functions in biofilm development (Colvin et al. 2012; Ma et al. 2009; Vasseur et al. 2005; Yang et al. 2011a; Zhao et al. 2013), increases in either (or both) of their promoter activities may enhance biofilm thickness, compactness, and survival. My experiments supported this hypothesis (Table 4.1). Moreover, these phenotypes were also enhanced by addition of nitrate, supporting the preferential advantage for utilizing an alternative terminal electron acceptor under hypoxic conditions of biofilm-growth.

5.3 Conclusion and Outlook

In this study, I investigated the function of two proteins, AlgL and MucR, in alginate production and biofilm formation. It was concluded that AlgL is likely to be a free periplasmic protein serving a maintenance role to degrade misguided alginate from the periplasm. In contrast, MucR, as a multimeric inner membrane protein, positively affects alginate production. However, when nitrate is present, MucR suppresses alginate biosynthesis at multiple levels through mechanisms dependent and independent on c-di-GMP. Furthermore, while AlgL has no effect on biofilm attachment, growth or dispersal, MucR and nitrate differentially affect expression of Psl and Pel production, attachment and biofilm formation.

In the context of managing *P. aeruginosa* infections, the apparent necessity of MucR and AlgL in producing alginate during planktonic mode (Wang et al. 2015; Wang et al. 2016a) – which is physiologically similar to the acute infection state – could be exploited by therapeutic interventions that interfere with their function. For instance, disabling either or both enzymes at early stages of infection might make it more difficult for *P. aeruginosa* to transition into a persistent/chronic alginate over-producing state that is much harder to treat. However, targeting these proteins in established biofilms might not be effective. Hence for established biofilms, nitrate (or denitrification intermediates) and exogenous lyases could be used to suppress alginate production (and degrade the biofilm matrix), making the organism more susceptible to antimicrobial agents and host immune responses.

In the context of using bacteria to make tailor-made alginates, expression of MucR with an inactive MHYT II motif or catalytically inactive AlgL may help increase alginate yields. While this information is derived from a potentially pathogenic microorganism,

similar regulatory mechanisms may be exploitable in non-pathogenic *Pseudomonas* or *Azotobacter* spp. to develop microbial alginate factories.

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Research outputs

Work from this thesis contributed to the follow research outputs:

Wang, Y, Moradali MF, Goudarztalejerdi A, Sims IM, Rehm BHA (2016) The Alginate Lyase (AlgL) is Dispensable for Alginate Production by *Pseudomonas aeruginosa*. Paper presented at the ASM Microbe 2016, Boston, USA.

Wang Y, Hay ID, Rehman ZU, Rehm BHA (2015) Membrane-anchored MucR mediates nitrate-dependent regulation of alginate production in *Pseudomonas aeruginosa*. *Appl Microbiol Biot* 99(17):7253-7265 doi:10.1007/s00253-015-6591-4.

Wang Y, Moradali MF, Goudarztalejerdi A, Sims IM, Rehm BHA (2016) Biological function of a polysaccharide degrading enzyme in the periplasm. *Sci Rep* 6 doi:10.1038/srep31249.

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