Regulation and export of alginate in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a clinically important opportunistic human pathogen which is of particularly relevance to cystic fibrosis (CF) patients where *P. aeruginosa* pulmonary infections are the leading cause of both morbidity and mortality.

The CF lung provides a unique environment to the pathogen which induces the overproduction of the exopolysaccharide alginate by the bacteria, resulting in a thick biofilm which protects the bacteria from the host immune response and antibiotic treatment, while contributing to the clogging of the lung. Furthermore, this switch from a non-mucoid (minimal levels of alginate) to a mucoid (alginate over-producing) phenotype is widely recognised as a poor prognosis indicator for patients, after which the infection cannot be eradicated. The exact mechanisms responsible for this switch are unclear but appear to involve a complex combination of transcriptional regulation, post translational regulation and the mutation of hyper-mutable regions of the genome.

This thesis investigates the physiological role of alginate for *P. aeruginosa* as well as several of the previously poorly understood steps in the biosynthesis and regulation of this important virulence factor. The outer membrane pore, AlgE, responsible translocation of alginate across the bacterial outer membrane was characterised. Interactions between two uncharacterised proteins, AlgK and AlgX, were identified and two novel regulatory networks involved in the control of alginate biosynthesis were identified.

Preface

The format of this thesis complies with the "Submission of thesis based on publications" as described in the latest version of the Handbook for Doctoral Studies, version 6, published by the Massey University doctoral research committee (January 2010).

The following sections of this thesis have been published or are submitted for publication in internationally peer-reviewed journals. Publications do not appear in chronological order.

Chapter I

Hay ID, Rehman ZU, Ghafoor A and Rehm BHA (2010). Bacterial biosynthesis of alginates. Journal of Chemical Technology and Biotechnology **85**: 752-759

Chapter II

Hay ID, Gatland K, Campisano A, Jordens JZ and Rehm BHA (2010). Impact of alginate production on attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa* strain. Applied and Environmental Microbiology **75**: 6022-6025

Chapter III

Hay ID, Rehman Z and Rehm BHA (2010). Membrane topology of outer membrane protein AlgE, which is required for alginate production in *Pseudomonas aeruginosa*. Applied and Environmental Microbiology **76**: 1806-1812

Chapter IV

Hay ID, Remminghorst U and Rehm BHA (2009). MucR, a novel membrane associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Applied and Environmental Microbiology **75**: 1110-1120

Chapter V

Hay ID, Schmidt O, Gutsche J and Rehm BHA (2011). Identification of a periplasmic AlgK-AlgX-MucD multiprotein complex in *Pseudomonas aeruginosa* involved in biosynthesis and regulation of alginate. Applied Microbiology and Biotechnology (accepted June 10 2011)

Contribution IDH made to publications by are as follows:

Chapter I: This review was drafted by IDH with critical review from ZUR & AG and finalised by BHAR.

Chapter II: Continuous flow biofilm growth, confocal microscopy and subsequent analysis were done by IDH (with assistance from KG). Solid surface attachment experiments were done by JZJ and AC.

Chapter III: *algE* deletion mutant and complementation vector were made by IDH. pEX100T:ΔalgE was made by Uwe Remminghorst. Construct expressing AlgE with deletion of extracellular loop 7 was made by IDH. FLAG tag insertions of algE were made by ZUR. Alginate quantification was done by ZUR. Outer membrane protein isolation and immunobloting was done by IDH. Manuscript was drafted by IDH , and finalised by BHAR.

Chapter IV: UR made the plasmids pEX100T:ΔmucR and pBBR1MCS-5:mucR. All other work was done by IDH. Manuscript was drafted by IDH and finalised by BHAR.

Chapter V: Plasmid pEX100T:ΔmucD was made by JG. Strains PAO1ΔmucD, PAO1ΔalgX, and PAO1ΔmucDΔalgX were made by OS. All other work was done by IDH. Manuscript was drafted by IDH and finalised by BHAR.

DNA sequencing and MALDI-TOF/MS were provided by external services.

This is to certify that the above mentioned work was conducted by Iain Hay.

Signature Date Signature Date

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Prof. Bernd H.A. Rehm Iain D. Hay

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"Extraordinary claims require extraordinary evidence"-Carl Sagan

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Chapter I

Introduction

General Introduction and overview

Pseudomonas aeruginosa is a commonly found, clinically important opportunistic human pathogen. *P. aeruginosa* infection are common in immune-compromised patients and are of particular concern to burns patients, patients with implanted medical devices such as catheters, and patients with cystic fibrosis. Among Cystic fibrosis patients, *P. aeruginosa* pulmonary infections are the leading cause of morbidity and mortality (31). The establishment of a chronic infection in the lungs of patients with CF coincides with the switch of *P. aeruginosa* from a so-called "non-mucoid" phenotype to a "mucoid" phenotype. The mucoid phenotype is characterised by overproduction of the exopolysaccharide alginate; this is typically a poor prognostic indicator associated with a decline of pulmonary function and survival rate for these patients (98, 118, 130).

Alginate functions as extracellular matrix material creating a favourable environment for the bacteria. Although not the only exopolysaccharide produced by *P. aeruginosa*, alginate overproduction enables the formation of thick biofilms with highly defined micro-colony structures. Alginate production is not absolutely required for *P. aeruginosa* biofilm formation (185), but previous studies have provided evidence that it plays a role maturation and development of biofilms (67, 113). Alginate production and the associated biofilms have been shown to be beneficial to the resident bacteria in several ways; alginate has been shown to prevent the diffusion of clinical antibiotics making the treatment and subsequent clearing of the infection doubtful (161). Furthermore alginate protects the bacteria from the host's immune system, it has been shown to prevent the uptake of *P. aeruginosa* by macrophages (119, 159), scavenge and protect the bacteria from toxic free radicles produced by the immune system (160), and inhibit a Th1 immune response (163).

The genetics and biochemical steps involved in alginate biosynthesis will be covered in more detail in the succeeding mini-review. Briefly, there are 13 core proteins responsible for the biosynthesis of alginate in *P. aeruginosa*, all but one of these proteins are encoded on a single operon. The initial cytosolic steps leading up to the polymerisation and secretion of alginate have been well characterised. The remaining proteins have been proposed to form a multi-protein complex spanning the inner membrane, the periplasm and into the outer membrane, though no direct experimental evidence supporting any protein-protein interaction or multi-protein complex has been demonstrated. The proteins responsible for the polymerisation and modification of the nascent alginate chain

have been identified, and characterised to some extent. The remaining steps in the translocation of alginate through the periplasm and across the outer membrane are relatively poorly understood.

AlgE is thought to be involved in secretion of alginate through the outer membrane; though the recombinant expression and subsequent biochemical and bioinformatic analysis of this protein strongly support this hypothesis its direct requirement for alginate polymerisation and or secretion has not been demonstrated (128, 130). Another protein, Alg44, has been shown to be essential for polymerisation through an unknown mechanism. Alg44 contains a recently identified PilZ domain which is essential for its function (102). The PilZ domain is involved in the sensing of the bacterial secondary messenger cyclic dimeric guanosine monophosphate (c-di-GMP). C-di-GMP is a central regulator of bacterial physiology; the regulatory networks responsible for c-di-GMP signalling are complex, diverse and poorly understood. The Alg44 PilZ domain provides the framework for a level of post translational regulation that warrants further investigation. Two further proteins required for the secretion of alginate are AlgX and AlgK. The function of these two proteins is unknown and they have no homologues or predicted domains that would provide insight in to their function. Purification of AlgX has provided some insights into its potential role, a regulatory protein, MucD, co-purified along with it. This apparent connection between a core alginate biosynthesis protein and a protein involved in the regulation of alginate warrants further investigation.

Several levels of regulation are involved in the regulation of alginate production in a physiological setting. The Regulation of alginate will be covered in more detail in the succeeding section. Briefly, the alginate operon is under the tight transcriptional control of a promoter upstream of *algD*, this promoter is controlled by the alternate sigma factor AlgU (σ^{22}, AlgT). AlgU is sequestered at the cytoplasmic membrane by the intramembrane anti-sigma factor MucA. MucA is controlled by a complex regulated intramembrane proteolysis (RIP) cascade involving at least 5 proteases, various extracellular stresses can "activate" this cascade that leads to the proteolysis of MucA and subsequent release of the AlgU sigma factor (37, 100, 152, 124. 182). Furthermore, the *mucA* gene and genes encoding related regulatory is prone to mutation and is a common site of mutations in clinical (mucoid) isolates, one study found that 80% of all clinical isolates contained mutations in this region, these hypermutable regions can be thought of as an additional level of regulation (17, 25). As discussed above an additional post-translational level of regulation is emerging with the discovery of the c-di-GMP binding site in Alg44.

Thesis Aims

The overall aims of this thesis are:

- To provide further insights in to the physiological role of alginate for *P. aeruginosa*
- Investigate the requirement and role of AlgE in the biosynthesis of alginate in *P. aeruginosa*
- Investigate the role of c-di-GMP in alginate biosynthesis and attempt to identify and characterise proteins involved
- Investigate the apparent connection between the core alginate proteins and the regulatory network that controls their expression
- Provide insights into the proposed multi-protein alginate biosynthesis complex

Thesis Findings

Chapter I of this thesis contains a literature review of the subject area. It is composed of a mini review published in the international peer reviewed Journal of Chemical Technology and Biotechnology.

Chapter II provides insights in to the role of alginate in biofilm formation. It was shown that although increased levels of alginate leads to more mature developed biofilms, it is inhibitory to the initial attachment of the bacteria to surfaces. This chapter was published in the international peer reviewed journal Applied and Environmental Microbiology.

Chapter III demonstrated the requirement of AlgE for the secretion of alginate but not for the polymerisation. Further insights in to the topology of AlgE are provided. This chapter was published in the international peer reviewed journal Applied and Environmental Microbiology.

Chapter IV investigates the role of c-di-GMP. A c-di-GMP producing protein that seems to specifically control the level of alginate biosynthesis was identified and characterised. This chapter was published in the international peer reviewed journal Applied and Environmental Microbiology.

Chapter V provided insights into the previously identified interaction between AlgX and MucD. An additional member of this complex was identified, AlgK. This is the first documented interaction between members of the alginate biosynthetic machinery.

Furthermore it was shown that there is a relationship between the stability of the proposed complex and the activation of the alginate promoter. This chapter was published in the international peer reviewed journal Applied Microbiology and Biotechnology.

Overall this thesis provided insights into the mechanisms behind the biosynthesis and regulation of a key physiological process by an important opportunistic human pathogen. Furthermore, many of the findings of this thesis provide insights into processes relevant to the broader microbiology community such as biofilm formation and bacterial signalling.

Literature Review:

Bacterial biosynthesis of alginates

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Abstract

Alginates are polysaccharides with many industrial and medical uses, from food additives to encapsulation agents in the emerging transplantation technologies. Alginate is composed of variable proportions of β-D-mannuronic acid and α-L-guluronic acid linked by 1–4 glycosidic bonds. Traditionally, commercial alginate has been produced by farmed brown seaweeds, but this alginate suffers from heterogeneity in composition and quality partly due to environmental variation. Two bacterial genera, *Pseudomonas* and *Azotobacter,* are also capable of producing alginate as an exopolysaccharide. These bacterial alginate producers can provide the means to produce alginates with defined monomer composition and possibly through genetic and protein engineering may allow for the production of 'tailor made' bacterial alginates. The paper discusses the mechanisms behind alginate production in bacteria and how they may be used in the commercial production of alginates.

Introduction

Alginate was first discovered in brown seaweed in the late 19th century. Since its discovery alginate has become an important industrial product, used in the food, material, cosmetic and medical/pharmaceutical industries. Alginate is a polysaccharide composed of variable proportions of 1,4-linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic (G), depending on its origin. Differences in the proportions and grouping of these monomers results in alginates with marked differences in their chemical and physical properties. Alginate is capable of forming gels in the presence of divalent cations (e.g. Ca^{2+}) and alginates with higher proportions of G blocks can bind cations more efficiently and thus, form more rigid gels. Additionally, bacterial derived alginates are acetylated at the O2/O3 positions of the mannuronate residues changing their material properties by increasing the water holding capacity. At present most commercial alginate is produced by farmed brown seaweeds (primarily *Laminaria hyperborean* and *Macrocystis pyrifera*). Alginates from these sources are often heterogeneous in composition and lacking the desired material properties (84, 108).

Two bacterial genera are also capable of producing alginate as an exopolysaccharide, *Pseudomonas* and *Azotobacter*. They produce alginates with marked differences in material properties and function. One of the model organisms for alginate production is the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is of great importance to cystic fibrosis (CF) patients, where it is the leading cause of morbidity and mortality. In *P. aeruginosa*, alginate has been shown to be important for the formation of thick highly structured biofilms (65, 113). The CF lung provides a unique environment to the pathogen, which induces the bacteria to overproduce alginate, producing a thick biofilm, protecting itself from the host immune response and antibiotic treatment, while contributing to the clogging of the lung (119, 159-161, 163). In *Azotobacter*, the alginate produced can have a higher concentration of G-blocks and thus is relatively stiff, this alginate is used in the formation of desiccation resistant cysts (144).

As alginates become used in increasingly more applications, both medical and industrial, it is becoming desirable to better control their material properties. Owing to a better understanding of the polymerization, modification, and regulation of alginate biosynthesis in these bacteria, and the relative ease of optimizing and modifying bacteria for production, bacterial alginates could perform where seaweed derived alginates underperform. Bacterial alginates may provide a base for the production of alginate with more defined chemical and material properties, furthermore, alginates could potentially

be tailor-made to have certain properties. Already, epimerases from *Azotobacter vinelandii*, which convert M residues to G residues, have be used to modify the G content, and thus the material properties of seaweed derived alginates (108), and alginates from mutant *Pseudomonas fluorescence* can be produced that lack any G residues (polymanuronan) (24, 53).

Genetics of Bacterial Alginate Biosynthesis

The work of Darzins and Chakrabarty (36) first demonstrated many of the genes involved in alginate production in *P. aeruginosa*, using complementation studies. To date at least 24 genes have been found to be directly involved in alginate production in *P. aeruginosa* (125) (Table 1). With the exception of *algC* all the structural genes involved in alginate biosynthesis are clustered in a single operon, first described by Chitnis and Ohman (25). The cluster consists of 12 genes: *algD, alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF*, and *algA* located at approximately 3.96 Mb on the PAO1 genome map. This operon is under the tight control of a promoter located upstream of *algD* (132, 151, 155). Within this operon are the genes encoding proteins involved in alginate precursor synthesis (*algD* and *algA*); proteins that modify the nascent alginate chain (*algI, algJ*, and *algF* for acetylation (50), *algG* for epimerization (47), and *algL* for degradation (149)); the putative outer membrane porin (*algE*) (128, 130). The products of the *alg8* gene are thought to be involved in the transfer of GDP-mannuronic acid across the cytoplasmic membrane and have recently been shown to play a role in the polymerization process (135). The product of the *alg44* gene is thought to be involved in the post-translational regulation of alginate (67, 135). The functions of the products of the remaining two genes in the operon, *algK* and *algX*, are unclear but their products are essential for production of alginate and are thought to play some sort of structural or protective role, guiding the alginate polymer through the periplasmic space (57, 75). Some of the genes involved in alginate production encode proteins that are not exclusively involved in alginate biosynthesis. This is true for the *algC* gene, encoding a phosphomannomutase which is involved in precursor synthesis. This gene product is also involved in rhamnolipid and lipopolysaccharide biosynthesis (54, 115, 188) and expression is driven from its own promoter (192). Also several of the regulatory proteins do not act exclusively on alginate biosynthesis genes, as will be discussed below. The multiple roles of these proteins would suggest that alginate production is part of a much larger, complex metabolic and regulatory network.

Table 1 Genes involved in alginate biosynthesis and their function (or putative function). Adapted from Rehm (2002) (125)

Gene	Gene product	Reference
algD	GDP mannose dehydrogenase	(167)
alg8	Glycosyltransferase/polymerase function?	(134)
alg44	c-di-GMP binding regulation/ membrane fusion?	(135)
algK	Periplasmic scaffold?	(75)
algE (algJ)	Outer membrane alginate porin?	(126, 128)
algG	Mannuronan C-5-epimerase	(47)
algE1-E7	Azotobacter extracellular epimerases	(39)
algX	Periplasmic unknown function/scaffold/sequesters MucD	(57)
algL	Alginate lyase	(76)
algI	O-Acetylation	(50)
algJ (algV)	O-Acetylation	(50)
algF	O-Acetylation	(50)
algA	Phosphomannose isomerase/GDP mannose pyrophosphorylase	(156)
algB	Member of ntrC subclass of two-component regulators	(94)
algC	Phosphomannomutase	(188)
algH	Unknown	(10)
algR	Regulatory component of two-component sensory transduction system	(94)
algQ	Histone like transcription regulator. AKA *algR2*	(78)
algP	Histone-like transcription regulator. AKA *algR3*	(79)
algZ	AlgR cognate sensor. AKA fimS (PA5262)	(7)
amrZ	Arc-like DNA binding protein. Formally called *algZ* (PA3385)	
algU	Homologue of *E. coli* σ^E global stress response factor/σ^{22}	(186)
mucA	Anti σ factor	(186)
mucB	Anti σ factor	(23)
mucC	Regulator?	(16)
mucD	Homologue of *E. coli* serine protease DegP	(181)
algW	Homologue of *E. coli* serine protease DegS	(23)
mucP	Homologue of *E. coli* RseP protease involved in AlgU RIP cascade	(123)
mucE	Periplasmic or outer membrane protein involved in AlgU RIP cascade	(123)
mucR	Alginate specific diguanylate cyclase (c-di-GMP synthesizing)	(67)

Biosynthesis of Alginate

The first bacterial alginate biosynthesis pathway was proposed in 1975 by Pindar and Bucke in *A. vinelandii* (120). A combination of complementation studies and overexpression studies has provided a convincing model for the biosynthesis of alginate in *P. aeruginosa*. This can be broken down into four stages: (i) precursor synthesis; (ii) polymerisation and cytoplasmic membrane transfer; (iii) periplasmic transfer and modification; and (iv) export through the outer membrane (137) (Fig. 1).

By far the best understood part of alginate biosynthesis in *P. aeruginosa* is the synthesis of the precursor GDP-mannuronic acid in the cytosol. Radio-labelling studies have shown that the synthesis starts with the entry of six carbon substrates to the Entner-Douderoff pathway (KDPG pathway), resulting in pyruvate, which is channelled towards the tricarboxylic acid (TCA) cycle, while oxaloacetate from the TCA cycle can be converted to fructose-6-phosphate via gluconeogenesis (93, 111). The conversion of fructose-6-phosphate to mannose-6-phosphate is catalysed by the phosphomannose isomerase (PMI) activity of the bifunctional protein AlgA (PMI-GMP) (101). AlgC (phosphomannomutase) directly converts mannose-6-phosphate to mannose-1-phosphate (191). The GDP-mannose pyrophorylase (GMP) activity of AlgA (PMI-GMP) catalyses the conversion of the activated mannose-1-phosphate to GDP-mannose with the hydrolysis of GTP (156). The GMP activity of this enzyme favours the reverse reaction, but the constant conversion of GDP-mannose to GDP-mannuronic acid by the activity of AlgD (GDP-mannose-dehydrogenase) shifts the reaction towards GDP-mannuronic acid and alginate production. This AlgD catalysed reaction is essentially irreversible and provides the direct precursor for polymerization, GDP-mannuronic acid (143). This and the high intracellular levels of GDP-mannose would indicate that this AlgD catalysed step is a limiting step and/or important kinetic control point in alginate biosynthesis (167).

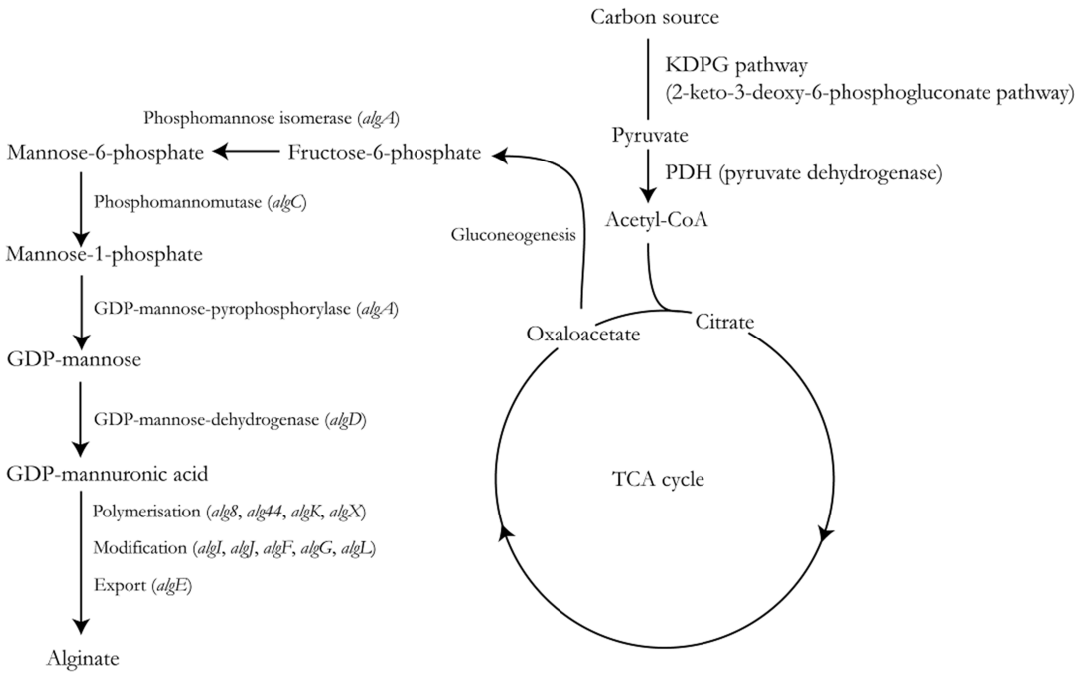

Figure 1. Proposed alginate biosynthesis pathway in *P. aeruginosa* (adapted from Remminghorst & Rehm, 2006).

Polymerization and translocation are the poorest understood processes in alginate biosynthesis. This is mainly due to the fact that at present no polymerase or polymerase complex has been purified, and up until recently no alginate synthesis activity had been demonstrated *in vitro* (137). Alg8 is thought to be a good candidate for the alginate polymerase enzyme. It shares homologies with class II glycosyltransferases, which catalyse the transfer of sugar residues from an activated donor to an acceptor molecule, which can be a growing carbohydrate chain (137, 148). Furthermore, structural similarities were observed when compared with functionally related enzymes such as AcsAB (cellulose synthase) (147) and Chs1 (chitin synthase) (137). Overproduction of Alg8 results in a 15-fold increase in the levels of alginate biosynthesis, this suggests that Alg8 is a key bottleneck and further suggests that it is the catalytic subunit of the multi-protein complex. AlgG, AlgK and AlgX are thought to provide some kind of periplasmic scaffold, along with the outer membrane protein AlgE, to guide and protect the nascent alginate chain from lyase degradation (1, 53, 66, 74, 138). Deletion mutants of these proteins did not show alginate production but showed secretion of free uronic acids, indicating that polymanuronate is being formed and subsequently degraded by the periplasmic alginate lyase (AlgL). This suggests that they are not playing a direct role in the polymerization process but may be playing more of a structural/protection from alginate lyase degradation (discussed below) role (53, 76, 138).

It has been suggested that Alg8 and Alg44 interact and play an important role in the polymerase complex (145). Recently, it was shown in complementation studies that Alg44 is required for alginate polymerization. Homology based secondary structure predictions showed some level of similarities of the C terminal half of Alg44 to the membrane bridging protein MexA. This suggests a function of Alg44 as part of the periplasmic scaffold, where it may provide a bridge between the cytoplasmic membrane protein Alg8 and the outer membrane export protein AlgE (135) (Fig. 2). Additionally the cytosolic N terminal half of Alg44 has been shown to possess a functional and essential PilZ domain (102, 135). PilZ domains are involved in binding of the regulatory molecule bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (3). This may suggest an additional regulatory role for Alg44, as shown previously for the cellulose synthase.

Modification of alginate in bacteria occurs almost exclusively in the periplasm, which suggests that alginate is synthesized as polymannuronate and that modification occurs at polymer level. Three classes of alginate modifying enzymes have been described: transacetylases (48-50, 157), mannuronan C5-epimerases (24, 47, 53), and lyases (2, 6). In *P. aeruginosa* AlgI, AlgJ (called AlgV in *Azotobacter*), and AlgF were shown to form the acetylation complex (48-50, 157). Transacetylation occurs at the O-2 and/or O-3 position and only occurs at mannuronic acid residues. Acetylation of these residues prevents their epimerization to guluronic acid residues by AlgG. It also prevents degradation of the alginate chain by AlgL (47, 49, 179). Thus, the acetylation of alginate indirectly controls epimerization and length of the alginate polymer.

Mannuronan C-5-epimerase introduces guluronic acid residues into the polymannuronate. There has only been one such enzyme identified in *P. aeruginosa*, AlgG, whereas *A. vinelandii* has a total of eight epimerases: the periplasmic AlgG and the extracellular AlgE1–AlgE7 (39, 129). These Ca^{2+} dependent extracellular epimerases have differing specificities for M G composition and introduce different distributions of guluronic acid residues into their substrate molecules, additionally AlgE7 has alginate lyase activity. This might explain the highly variable alginate produced by *A. vinelandii*, which is important for cyst formation and differentiation (39, 71, 144).

AlgL, catalyses the β-elimination reaction leading to the degradation of alginate. The exact biological function of alginate lyase has not been fully understood but alginate-producing bacteria are not able to reutilize the alginate as a carbon source. There is conflicting evidence as to the necessity of AlgL in the biosynthesis of alginate: Albrecht and Schiller (2) demonstrated that loss of the *algL* gene resulted in a complete loss of alginate

biosynthesis which could be restored by complementation with the *algL* ORF in trans but not with an ORF encoding AlgL variants lacking lyase activity (containing mutations in the putative active regions); whereas Jain and Ohman (76) showed that loss of *algL* does not result in loss of alginate biosynthesis, but leads to accumulation of alginate in the periplasm and eventually the lysis of the cells. Additionally AlgL has been shown to be dispensable for both alginate biosynthesis and growth in *Azotobacter vinelandii* (172). It has been suggested that AlgL may function in controlling the length and molecular weight of the alginate polymer, providing short alginate polymers to 'prime' the polymerization of alginate (19), or to clear the periplasm of misguided alginate (6, 76). It should be noted that in addition to AlgL and its orthologues found in all alginate-producing bacterial species, additional alginate lyase proteins have been identified in both alginate-producing and non-producing bacteria (for review see Wong, T.Y *et al.* (179)). These enzymes have differing residue specificities and cellular localizations. Among *Pseudomonas* species there has been one other protein identified to have alginate lyase activity, PA1167 (187). Among *Azotobacter* species at least four other lyases have been identified: the epimerase AlgE7, and three others, AlyA1, AlyA2 and AlyA3 (52).

AlgE (called AlgJ in *Azotobacter*) is the putative porin through which the alginate is secreted. Electrochemical analysis of AlgE showed that it functions as an anion-selective pore (128). This protein has been shown to be essential for the secretion of intact alginate and can be detected in the outer membrane of mucoid, alginate-overproducing, strains of *P. aeruginosa* but is absent in non-mucoid strains (55).

Regulation of Bacterial Alginate Biosynthesis

The regulation of alginate biosynthesis is complex and involves transcriptional and post-translational regulation, as well as several hypermutable regions of the genome which can switch on or off alginate production. Both alginate biosynthesis specific regulators and globally acting regulators influence alginate biosynthesis. Transcriptional regulation of alginate biosynthesis in *P. aeruginosa* can be loosely divided into two different types: environmental stimuli based regulation; and a 'genotypic switch' based form of regulation.

The 'switch' loci, contains the genes *algU, mucA, mucB, mucC* and *mucD. algU* encodes an extra-cytoplasmic alternate σ^{22} factor, which is at the apex of a hierarchy of regulators involved in alginate biosynthesis and is ultimately required for transcription starting from the AlgD promoter and thus expression of the core alginate genes (25, 37, 45, 124). This region shows high similarity to the well-characterized σ^{E} region in *E. coli*, containing the

genes *rpoE* (encoding the σ^E), *rseA, rseB, rseC*. This operonic structure appears to be conserved among most of the gamma proteobacteria (180). In *E. coli* the σ^E signal transduction pathway is part of an envelope stress response system, whereby external stresses such as temperature fluctuations are thought to lead to misfolding of outer membrane proteins in the periplasm. This leads to activation of the sigma factor by a regulated intramembrane proteolysis (RIP) cascade (142). It is thought it functions in a similar way in *Pseudomonas*. The key proteins of the *Pseudomonas* alg/muc RIP cascade were elucidated over a decade ago. These are the sigma factor AlgU (σ^{22}) and the intramembrane anti-sigma factor MucA. AlgU is required for transcription of the alginate operon as well as transcription several of the regulatory operons (including its own). MucA inhibits the transcriptional activation activity of AlgU by sequestering it at the cytosolic side of the inner membrane preventing it from binding to RNA polymerase (100, 151, 152, 186). The periplasmic protein MucB has been shown bind to the periplasmic side of MucA and play a negative regulatory role in alginate biosynthesis by protecting MucA from proteolysis and aiding in the sequestering of AlgU (23, 182). It has been shown that mutation of both MucA and/or MucB leads to transcriptional activation of the *algD* promoter and a highly mucoid (alginate overproducing) phenotype (97, 100), indicating that the cytosolic AlgU sequestering activity of MucA is dependent on the periplasmic MucB protein. Several of the proteases involved in the RIP cascade degradation of MucA have recently been identified, Wood and Ohman (23, 182) demonstrated that in response to envelope stress MucA is initially cleaved by the periplasmic protease AlgW (*E. Coli* DegS homologue) and subsequently cleaved on the cytosolic side by the intramembrane protease YaeL (E. coli RseP/YaeL homologue) leading to the release of AlgU. The PDZ activating domain (de-repression) of AlgW is activated by C-terminus of particular misfolded proteins, in particular a periplasmic or outer membrane located protein called MucE (123). A third periplasmic protease, MucD, appears to be play a role antagonistic to that of AlgW. Deletion mutations of MucD lead to a mucoid phenotype indicating a negative regulatory role (181). MucD appears to be involved in the degradation of misfolded proteins in the periplasm that would otherwise activate (de-repress) AlgW (123, 182) (Fig.2). The role of MucC remains unclear. It should be noted that the alternate sigma factor, AlgU, released in this RIP cascade does no exclusively activate transcription of the alginate operon but is involved in the the transcription of other genes with diverse functions (44, 45).

There appears to be an addition level of 'control' over this cascade: one study found that over 80% of mucoid *P. aeruginosa* isolates obtained from CF patients contained mutations

in the *mucA* gene (17). Most of these mutations result in a premature stop codon and a truncated MucA. The most common mutation found in *mucA* was the deletion of a single guanine in a homopolymeric stretch of five G residues, resulting in a frame shift and subsequent recognition of a premature stop codon (mucA22 allele). Removal of MucA, the anti-sigma factor at the base of this complex RIP cascade means there is nothing to sequester the sigma factor AlgU and thus transcription from the *algD* promoter can proceed.

In addition to AlgU, several other proteins are required to initiate transcription of the alginate operon. AlgR is a response regulator part of a two component regulator that binds to three sites in the *algD* promoter, the sensory component of this regulatory pair is AlgZ (FimS) and strangely is not required for transcription of the alginate operon (94). AlgB is also part of a two component regulator and binds to one site on the *algD* promoter, again it is activity is apparently independent of its sensor kinase KinB (94). AmrZ (originally called AlgZ), an Arc-like DNA binding protein, binds to one site on the *algD* promoter (7, 8). These proteins are all also involved in the regulation of other diverse processes.

Recently a level of post-translational regulation of alginate biosynthesis has emerged. The alginate biosynthesis protein Alg44 contains a c-di-GMP binding/sensing PilZ domain in its C–terminus, indicating that c-di-GMP may be playing a regulatory role in alginate biosynthesis (3, 102). C-di-GMP is an important bacterial secondary messenger that has been linked to the post-transcriptional regulation of diverse processes such as motility, exopolysacharide production and virulence. Recently it has been demonstrated that one particular c-di-GMP synthesizing protein, MucR, specifically influences the levels of alginate biosynthesis in *P. aeruginosa* (67) (Fig. 2).

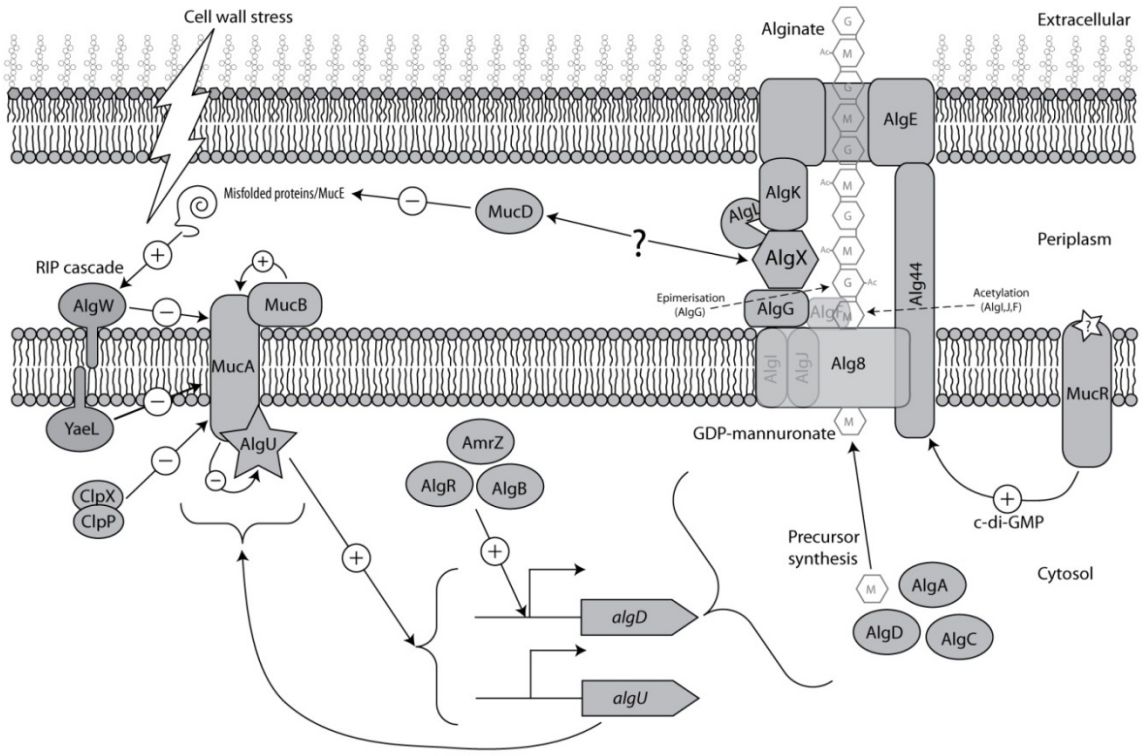

Figure 2. Overview of alginate biosynthesis in *P. aeruginosa*.

Applications of Bacterial Alginates

Since its discovery alginate has been used for many commercial purposes. Owing to its water holding, gelling, viscosifying and stabilizing properties, alginate of algal origin is used extensively in the food industry as a food additive. Based on its high biocompatibility (83), abundance and availability of the source, and relatively low cost to produce, alginates are increasingly being used in the medical field (168). Alginate dressings are widely used to treat exuding wounds. These dressings are generally comprised of fibres of stiff calcium alginate, which soften and can 'mould' to the area applied. In contrast to traditional wound dressings which adhere to a wound and can be painful and difficult to remove and reapply, these dressings do not adhere to the wound and can be removed with a saline wash (168). Alginate has also been used for the controlled release of drugs; the drugs are trapped in alginate beads, from which the drug can by slowly released (133). A similar method has been applied to encapsulate functional cells to be transplanted in to a subject (iso-, allo- or xenotransplantation) making the cells much less immunoreactive and thus less likely to be rejected by the subject, for example parathyroid tissue to treat hypoparathyroidism (60-63, 82). Recently alginate encapsulation has been used successfully as a method to orally deliver DNA based vaccines (169). To date the vast majority of the alginate used for commercial and medical purposes has been obtained via the harvesting of brown

seaweeds. These naturally occurring alginates are poor with respect to their purity and consistency of polymer composition. They are often contaminated with protein and other immunogenic compounds that require extensive downstream processing to remove. The relatively high price of the commercial production of bacterial alginates cannot compete with the low price of seaweed derived alginates. The potential for bacteria to produce high quality alginates with defined material properties for use in high value applications, such as those in the medical field, may provide a niche for the commercial production of bacterial alginates. Many of the material properties of alginate depend on the monomer composition, the degree of acetylation, or the length of the polymer as well as the degree and type of modifications. A knowledge of the mechanisms of the bacterial alginate modifying enzymes combined with the potential to manipulate and exploit these enzymes by genetic and protein engineering may allow for the production of 'tailor made' bacterial alginates with 'user defined' material properties (132, 145). These alginates could be produced directly from the bacteria or produced from the cheaper seaweed source or as polymannuron from *P. fluorescens* (24, 53), and later modified with bacterial enzymes. Indeed this has already been demonstrated with the use of extracellular epimerases secreted by *Azotobacter vinelandii* to treat polymannuron to produce alginates not found in nature with useful material properties (107, 108).

Chapter II

The impact of alginate overproduction on attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa*

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Abstract

The supermucoid *Pseudomonas aeruginosa* strain PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) showed strongly impaired attachment compared with the respective mucoid or nonmucoid strains and formed a thicker biofilm with large extended mushroom-like microcolonies. Alginate lyase treatment dissolved microcolonies. The data suggested that alginate overproduction impairs attachment but plays a structural role in microcolony formation.

Introduction

Alginate is an important virulence factor for *Pseudomonas aeruginosa*, and the conversion of nonmucoid strains to alginate-overproducing mucoid strains early after the infection of cystic fibrosis patients is associated with a decline of pulmonary function and survival rate (118, 130). Alginate functions as extracellular matrix material, enabling the formation of differentiated biofilms in which the diffusion of clinical antibiotics is decreased and the embedded cells are protected against human antibacterial defence mechanisms (113, 119). Although alginate is not required for *P. aeruginosa* biofilm formation (185), previous studies have provided evidence that it plays a role in the formation of thick and three-dimensional biofilms (67, 113). To further investigate the impact of alginate on attachment and biofilm architecture, we used a recently generated supermucoid strain, PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) (137). This strain showed about 15-fold alginate overproduction compared to alginate-producing mucoid *P. aeruginosa*. The gene *alg8* encodes the proposed catalytic subunit of alginate polymerase and is essential for alginate biosynthesis (137).

Quantitative analysis of attachment and biofilm formation

The attachment characteristics of the supermucoid strain PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) were compared with those of the wild-type strain PAO1 and the mucoid strain PDO300 (an isogenic *mucA22* mutant of PAO1) (99) and its alginate-negative isogenic *alg8* deletion mutant (137). A modification of the solid-surface assay (SSA) (116) was used to assess attachment in microtiter plates after incubation for 2, 4, and 6 h. Stationary cultures at 37°C in Luria-Bertani medium (containing gentamicin at 300 μg/ml when appropriate) were adjusted to an optical density at 600 nm of 0.05, and 100-μl aliquots were added to one column (8 wells) of each of five replicate 96-well tissue culture plates. After incubation at 37°C for the respective times, nonadherent bacteria were washed off by filling the wells three times with sterile water and then removing the well contents with gentle suction. Plates were then air dried, and adherent bacteria were stained with 100 μl of 0.1% (wt/vol) crystal violet for 20 min at room temperature. The crystal violet was removed by washing as described above and dissolved in 100 μl dimethyl sulfoxide. After 20 min, the absorbance at 595 nm was measured. The data presented here are the averages of results from three independent experiments with eight replicates each. The results showed excellent intra-assay and interassay reproducibility, with minimal background. During the early attachment phase (2 to 4 h), the nonmucoid strains (wild-type strain PAO1 and PDO300Δ*alg8*) showed significantly more attachment than any of the other strains (Fig.

1A). The supermucoid strain PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) showed the weakest attachment at 2, 4, and 6 h but wild-type levels of attachment and biofilm formation after 4 days (Fig. 1B). Biofilm analysis after stringent (41) or gentle (see description above) washing was used to evaluate the strength of the attachment of the grown biofilm. The difference between the effects of stringent and gentle washing was minimal for the PDO300Δ*alg8* mutant biofilm, where the extracellular matrix lacked alginate, while the greatest differences were observed in the alginate-rich biofilms of PDO300 and the supermucoid strain (Fig. 1B). These results suggest that alginate interferes with early attachment but subsequently promotes the formation of a thick, although loosely attached, biofilm.

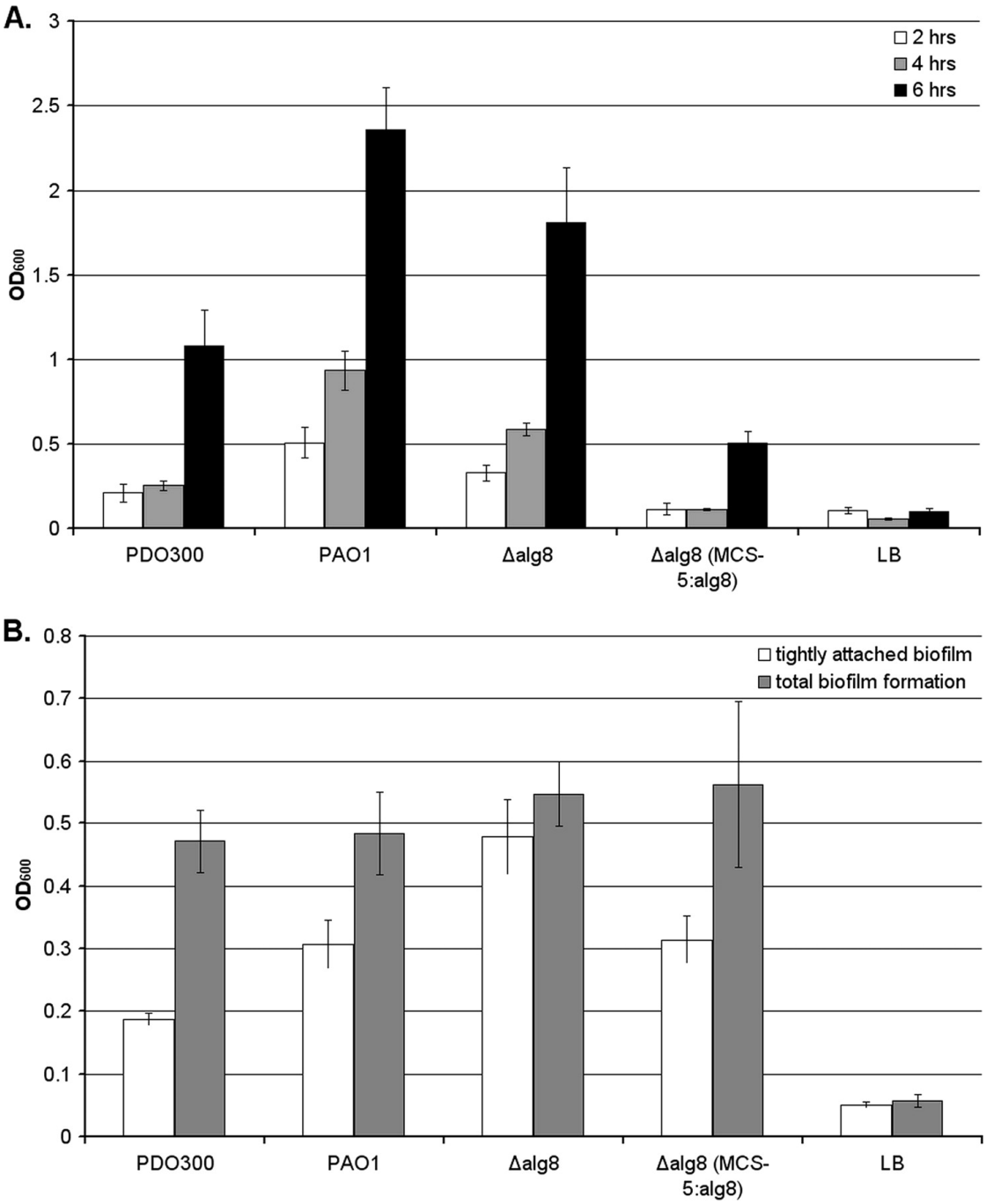

Figure 1. SSA analysis of various *P. aeruginosa* strains. PDO300, *P. aeruginosa* PDO300; PAO1, *P. aeruginosa* PAO1; Δ*alg8*, *P. aeruginosa* PDO300Δ*alg8*; Δ*alg8*(MCS-5:*alg8*), supermucoid strain; LB, uninoculated Luria-Bertani medium control; OD_{600}, optical density at 600 nm. (A) Differences during early attachment phase; (B) differences between loosely and tightly attached 4-day-old biofilms (adherent biofilms after gentle and stringent washing, respectively). Values and error bars represent the averages and standard deviations, respectively, for eight independent replicates.

Biofilm analysis

The morphology and architecture of biofilms of strains PDO300(pBBR1MCS-5) and PDO300Δ*alg8*(pBBR1MCS-5) and the supermucoid strain PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) were analysed in a continuous-culture flow chamber as described previously (22). After 4 days of growth in *Pseudomonas* isolation medium (137), biofilms were stained with dye from the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes Inc.) as described previously (22) and observed under a confocal laser scanning microscope (Leica TCS 4D) using the appropriate filters. Image analysis was performed and measurements were compiled using Imaris software (Bitplane Inc.). Strain PDO300(pBBR1MCS-5) showed a mature, fully differentiated biofilm consisting of well-defined finger-like microcolonies ranging in height from 75 to 145 μm with a diameter of 15 to 35 μm. The base layer of the biofilm ranged in thickness from 10 to 35 μm. The volume of this biofilm was 24.30 ± 3.78 $\mu m^3/\mu m^2$ (Fig. 2A and D). Strain PDO300Δ*alg8*(pBBR1MCS-5) showed a thinner, more uniform biofilm with no apparent microcolonies and a thickness ranging from 15 to 35 μm. The volume of this biofilm was 21.90 ± 5.76 $\mu m^3/\mu m^2$. Interestingly, the surface of this biofilm was visibly rougher and small clusters of cells were distinguishable, while both PDO300 and the supermucoid strain cells were embedded in the matrix and indistinguishable (Fig. 2A and C). The supermucoid strain PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) showed the thickest biofilm, ranging from 90 to 145 μm in height and consisting of large (up to 100-μm-diameter) spherical microcolonies. The concentrations of cells appeared to be highest at the bases of the microcolonies and decreased toward the tops of the microcolonies, where the edges became loosely defined and individual cells could be detected. Presumably this region contained high concentrations of alginate, keeping the cells associated with the microcolony. The base layer of the biofilm was also significantly thicker than those of the other biofilms, ranging from 35 to 55 μm. The volume of this supermucoid strain biofilm was 2.4 times that of the mucoid PDO300 strain biofilm, at 57.37 ± 4.34 $\mu m^3/\mu m^2$ (Fig. 2C and F). Proportions of dead and live cells did not differ among the tested strains (data not shown).

Mature biofilms were treated with a 100-μg/ml (1-U/ml) solution of alginate lyase from *Flavobacterium* sp. by exposure to a laminar flow (0.3 ml min^{-1}). This treatment showed no apparent effect on the PDO300(pBBR1MCS-5) and PDO300Δ*alg8*(pBBR1MCS-5) biofilms, whereas the supermucoid PDO300Δ*alg8*(pBBR1MCS-5:*alg8*) biofilm showed the large microcolonies dissolving from their tops and cells being released (see Movies S1 to S3 in the supplemental material). The same treatment with DNase or buffer alone did not cause any apparent dissolving or cell release (data not shown).

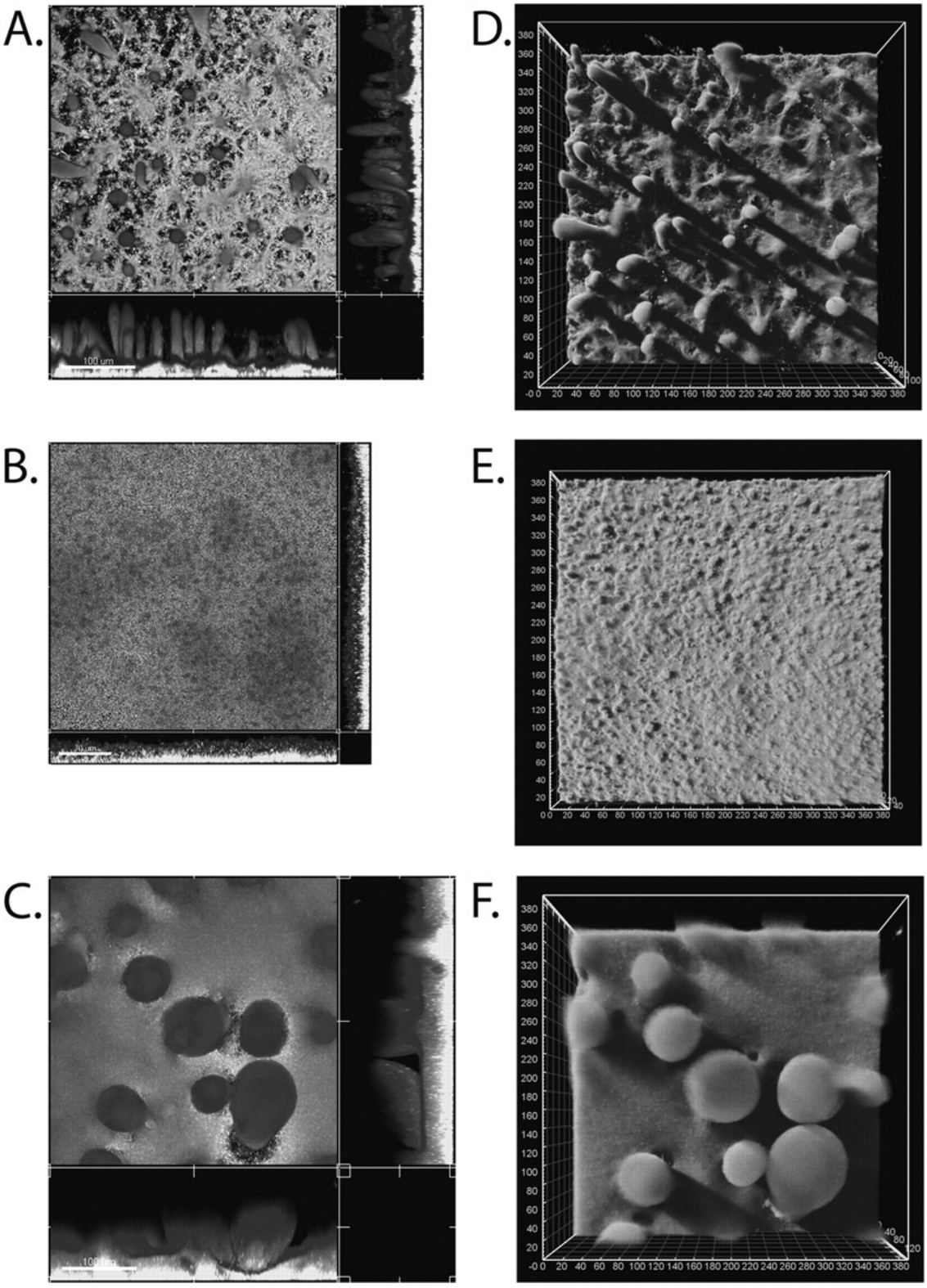

Figure 2. *P. aeruginosa* biofilm formation in a continuous-culture flow cell 4 days postinoculation as analyzed using a confocal laser scanning microscope. (A and D) *P. aeruginosa* PDO300(pBBR1MCS-5); (B and E) *P. aeruginosa* PDO300Δ*alg8*(pBBR1MCS-5); (C and F) *P. aeruginosa* PDO300Δ*alg8*(pBBR1MCS-5:*alg8*). Panels A, B, and C are sectional views; panels D, E, and F are three-dimensional projections. Images are representative of results from three independent experiments. The magnification is x400.

Discussion

In this study, the role of alginate in attachment and biofilm formation was studied using a recently genetically engineered supermucoid strain, PDO300Δ*alg8*(pBBR1MCS-5:*alg8*), which produces 15 times more alginate, when grown as a biofilm, than the already alginate-overproducing mucoid strain PDO300 (137). Exopolysaccharides produced by *P. aeruginosa* have been shown previously to be involved in attachment and biofilm formation (22, 73, 113, 116). Alginate has been shown to play a role in attachment to surfaces (95). However, the SSA analysis in this study clearly suggested that alginate overproduction impairs attachment to solid surfaces (Fig. 1A), as the mucoid strains showed the weakest attachment whereas the alginate-negative strain and the nonmucoid wild type showed the strongest attachment when assayed 2 to 6 h after inoculation (Fig. 1). Although PAO1 and PDO300Δ*alg8* are both nonmucoid, it should be noted that they showed some differences in the SSA which may be due to the PDO300 strain's defective *mucA22* allele, allowing the unrestricted activity of the AlgU sigma factor and thus promoting the transcription of not only the alginate biosynthesis operon but also other genes (45). Nivens and colleagues (113) provided evidence that alginate contributes to the biofilm architecture. Our results support this hypothesis; the finger-like microcolonies present in the mucoid strain biofilm were completely absent in the alginate-negative mutant biofilm (Fig. 2A, B, D, and E), indicating that alginate is essential for the formation of these biofilm structures. Additionally, it was shown that further increasing the levels of alginate production leads to the formation of extremely large microcolonies, demonstrating the important role this exopolysaccharide has in microcolony formation (Fig. 2C and F).

Treatment of the supermucoid biofilm with alginate lyase resulted in the breakdown of the microcolony structure, but this treatment had little effect on the mucoid and nonmucoid biofilms. This finding confirms that the supermucoid microcolonies were composed of high proportions of alginate. It is unclear why the presumably alginate-containing mucoid biofilm was not dissolved by the alginate lyase treatment in the same way. This outcome may be due to the lower proportion of alginate and, thus, the higher proportion of other biofilm matrix components present in the microcolonies, maintaining the biofilm structure.

The most striking difference found in the biofilm architecture of the supermucoid strain was the extensive formation of large, alginate-dense (as demonstrated by the alginate lyase treatment) microcolonies, at the tops of which individual cells could be detected,

suggesting a function for alginate in microcolony structure with an impact on the dispersal of cells from the biofilm as discussed elsewhere (18).

Acknowledgments

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Chapter III

Membrane topology of the outer membrane protein AlgE which is required for alginate production in *Pseudomonas aeruginosa*

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Abstract

The ubiquitous opportunistic human pathogen *Pseudomonas aeruginosa* secretes a viscous extracellular polysaccharide, called alginate, as a virulence factor during chronic infection of patients with cystic fibrosis. In the present study, it was demonstrated that the outer membrane protein AlgE is required for the production of alginate in *P. aeruginosa*. An isogenic marker-free *algE* deletion mutant was constructed. This strain was incapable of producing alginate but did secrete alginate degradation products, indicating that polymerization occurs but that the alginate chain is subsequently degraded during transit through the periplasm. Alginate production was restored by introducing the *algE* gene. The membrane topology of the outer membrane protein AlgE was assessed by site-specific insertions of FLAG epitopes into predicted extracellular loop regions.

Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic human pathogen responsible for chronic infections of the lungs of patients with cystic fibrosis (CF), in whom it is the leading cause of mortality and morbidity (31). The establishment of a chronic infection in the lungs of patients with CF coincides with the switch of *P. aeruginosa* to a stable mucoid variant, producing copious amounts of the exopolysaccharide alginate; this is typically a poor prognostic indicator for these patients (98, 130). Alginate is a linear unbranched exopolysaccharide consisting of 1,4-linked monomers of β-D-mannuronic acid and its C-5 epimer, β-L-guluronic acid, which is known to be produced by only two bacterial genera, *Pseudomonas* and *Azotobacter* (136). The switch to a mucoid phenotype coincides with the appearance of a 54-kDa protein in the outer membrane; this protein has been identified and has been designated AlgE (55, 130).

The genes encoding the alginate biosynthesis machinery are located within a 12-gene operon (*algD-alg8-alg44-algK-algE-algG-algX-algL-algI-algJ-algF-algA*). AlgA and AlgD, along with AlgC (not encoded in the operon), are involved in precursor synthesis (136). Alg8 is the catalytic subunit of the alginate polymerase located at the inner membrane (137). AlgG is a C-5 mannuronan epimerase (74). AlgK contains four putative Sel1-like repeats, similar to the tetratricopeptide repeat motif often found in adaptor proteins involved in the assembly of multi-protein complexes (11, 33). AlgX shows little homology to any known protein, and its role is unclear (57). Knockout mutants of AlgK, AlgG, and AlgX have nonmucoid phenotypes, although they produce short alginate fragments, due to the activity of the alginate lyase (AlgL), which degrades the nascent alginate (2, 57, 74-76, 138). AlgF, AlgI, and AlgJ are involved in acetylation of alginate, but they are not ultimately required for its production (50). The membrane-anchored protein, Alg44, is required for polymerization and has a PilZ domain for the binding of c-di-GMP, a secondary messenger essential for alginate production (67, 102, 135). The periplasmic C terminus of Alg44 shares homology with the membrane fusion proteins involved in the bridging of the periplasm in multidrug efflux pumps (38, 170). The periplasmic alginate lyase, AlgL, appears to be required for the translocation of intact alginate across the periplasm (2, 106). AlgE is an outer membrane, anion-selective channel protein through which alginate is presumably secreted (128). A protein complex or scaffold through which the alginate chain can pass and be modified and which spans the periplasm bridging the polymerase located (Alg8) at the outer membrane pore (AlgE) has been proposed (76). Indeed, it has been demonstrated that both the inner and the outer membranes are required for the *in vitro* polymerization of alginate (137).

The requirement of AlgE for the biosynthesis of alginate in *P. aeruginosa* was first observed by complementation of an alginate-negative mutant derived by chemical mutagenesis with a DNA fragment containing *algE* (29) Secondary structure predictions suggested that AlgE forms an 18-stranded β barrel with extended extracellular loops. Several of these loops show high densities of charged amino acids, suggesting a functional role in the translocation of the anionic alginate polymer (126, 128). Preliminary analysis of AlgE crystals has been reported (177).

In this study, the role of AlgE in alginate biosynthesis was investigated and the membrane topology of AlgE was assessed by site-directed insertion mutagenesis.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains, plasmids, and oligonucleotides used in the present study are listed in Table 1. The *Escherichia coli* strains were grown in LB medium at 37°C. *E. coli* S17-1 was used for conjugative transfer of suicide plasmid pEX100T:Δ*algE*Gm and flipase-encoding plasmid pFLP2. When they were required, the following antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; gentamicin, 10 μg/ml; and streptomycin, 30 μg/ml. The *P. aeruginosa* strains were grown in LB medium or *Pseudomonas* isolation agar (PIA) medium (20 g of peptone, 10 g of K_2SO_4, 1.4 g $MgCl_2$, 0.025 g of Triclosan, 20 ml of glycerol per litre) at 37°C, and when they were required, antibiotics were added at the appropriate concentrations. The antibiotic concentrations used for the *P. aeruginosa* strains were as follows: gentamicin, 300 μg/ml, and carbenicillin, 300 μg/ml. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Table 1: Bacterial strains, plasmids and oligonucleotide used in this study

Strain or plasmid	Characteristics	Source
Strains		
P. aeruginosa		
PDO300	*mucA22* isogenic mutant derived from PAO1	(72)
PDO300ΔalgE	Isogenic *algE* deletion mutant derived from PDO300	This study
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi-1 proA hsdR17* (r_{K^-} m_{K^+}) *recA1*; *tra* gene of plasmid RP4 integrated in chromosome	
Plasmids		
pGEM®-T Easy	Ap^r, P_{lac}	Invitrogen
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	
pBBR1MCS-5:*algE*	HindIII-BamHI fragment comprising *algE* inserted into vector pBBR1MCS-5	This Study
pBBR1MCS-5:*algE*DelL7	*algE* fragment with 57 bp deletion in region encoding putative surface exposed loop 7 inserted in to pBBR1MCS-5	This Study
pBBRMCS-5:*algE*L1FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 189th bp of the ORF	This Study
pBBRMCS-5:*algE*L2FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 336th bp of the ORF	This Study
pBBRMCS-5:*algE*L3FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 468th bp of the ORF	This Study
pBBRMCS-5:*algE*L4FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 582nd bp of the ORF	This Study
pBBRMCS-5:*algE*L5FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 711th bp of the ORF	This Study
pBBRMCS-5:*algE*L6FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 882nd bp of the ORF	This Study
pBBRMCS-5:*algE*L7FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1035th bp of the ORF	This Study
pBBRMCS-5:*algE*L8FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1269th bp of the ORF	This Study
pBBRMCS-5:*algE*L9FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1428th bp of the ORF	This Study
pEX100T	Ap^r Cb^r, gene replacement vector containing *sacB* gene for counterselection	(69)
pEX100T:Δ*algE*Gm	Ap^r Cb^r Gm^r; vector pEX100T with SmaI-inserted *algE* deletion construct	This Study
pPS865	Ap^r Gm^r; source of 1,100-bp BamHI fragment comprising *aacC1* gene flanked by Flp recombinase target site signal sequences	(69)
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(69)

Isolation, analysis, and manipulation of DNA. General cloning procedures were performed as described previously (146). All pBBR1MCS-5-derived plasmids were transferred to *P. aeruginosa* strains via electroporation, as described previously (27). DNA primers, deoxynucleoside triphosphates, and *Taq* and platinum *Pfx* polymerases were purchased from Invitrogen. The DNA sequences of the plasmid constructs were confirmed by DNA sequencing.

Construction and confirmation of *algE* deletion mutants. Two regions of the *algE* gene were amplified by using *Taq* polymerase and primers algE1N-Ec5, algE1C-Ba, algE2N-Ba, and algE2C-Ec5. Region algEN (469 bp) comprised bases 136 to 585 and region algEC (445 bp) comprised bases 1047 to 1472 relative to the designated *algE*-coding region (164). Both PCR products were hydrolysed by using BamHI and were inserted into the pGEM-T Easy vector (Promega). Vector pPS856 (69) was hydrolysed with BamHI. The fragment containing the *aacC1* gene (which encodes gentamicin acetyltransferase) flanked by two Flp recombinase target sites was inserted into the BamHI site of plasmid pGEM-TEasy:algENC, resulting in plasmid pGEM-TEasy:Δ*algE*Gm. The DNA of the 1,989-bp algEGm fragment was amplified by using *Pfx* polymerase and primers algE1N-Ec5 and algE2C-Ec5, and the corresponding PCR product was inserted into SmaI site of vector pEX100T (69), resulting in plasmid pEX100T:Δ*algE*ΩGm.

E. coli S17-1 was used as the donor for the transfer of plasmid pEX100T:Δ*algE*ΩGm into the *P. aeruginosa* strains, and transconjugants were selected on mineral salt medium (150) containing gentamicin and 5% (wt/vol) sucrose. Cells growing on this selective medium should have emerged from double-crossover events. Gene replacement was confirmed after subculture of the cells on PIA medium containing gentamicin and by PCR with primers algEup and algEdown.

E. coli S17-1 was used to transfer the Flp recombinase-encoding vector pFLP2 (69) into *P. aeruginosa* PDO300Δ*alg8*ΩGm strains, and after 24 h of cultivation on PIA medium containing 5% (wt/vol) sucrose, the gentamicin- and carbenicillin-sensitive cells were analysed by PCR for the loss of the gentamicin resistance-conferring cassette.

Complementation of isogenic *algE* deletion mutants. For complementation of the *algE* deletion mutants, the *algE* open reading frame (ORF) of *P. aeruginosa* PAO1 was amplified by using *Pfx* polymerase and primers algEN(HiSDNd) and algEC(Ba). This product was then inserted into the pGEM-T Easy vector, resulting in plasmid pGEMTEasy:*algE*. The *algE* fragment was released by hydrolysis with HindIII and BamHI

and was inserted into the HindIII and BamHI sites of broad-host-range vector pBBR1MCS-5 (85), resulting in plasmid pBBR1MCS-5:*algE*. In addition, a plasmid encoding AlgE with a 9-amino-acid deletion in the highly charged putative surface-exposed loop 7 region was constructed. This was constructed by amplifying the region upstream of the deletion point by using *Pfx* polymerase and primers algEN(HiSDNd) and algEDel7N(Ba) and the region downstream of the deletion point by using primers algEDel7C(Ba) and algEC(SaI). The two products were hydrolyzed with HindIII and BamHI or BamHI and SacI, respectively, and inserted into the HindIII and SacI sites of pBBR1MCS-5, resulting in plasmid pBBR1MCS-5:*algE*DelL7.

In order to probe the membrane topology of AlgE, insertions of the FLAG (DYKDDDDK) epitope were introduced into all of the nine putative surface-exposed loops. The insertions were constructed by site-directed, ligase-independent mutagenesis (SLIM) (26). Briefly, two PCRs were completed for each construct by using plasmid pGEMTEasy:*algE* as the template: one with the "FFLAG" and the corresponding "RS" primer (algEL1FFLAG and algEL1RS, algEL2FFLAG and algEL2RS, algEL3FFLAG and algEL3RS, algEL4FFLAG and algEL4RS, algEL5FFLAG and algEL5RS, algEL6FFLAG and algEL6RS, algEL7FFLAG and algEL7RS, algEL8FFLAG and algEL8RS, and algEL9FFLAG and algEL9RS) and one with the FS and the corresponding RFLAG primer (algEL1FS and algEL1RFLAG, algEL2FS and algEL2RFLAG, algEL3FS and algEL3RFLAG, algEL4FS and algEL4RFLAG, algEL5FS and algEL5RFLAG, algEL6FS and algEL6RFLAG, algEL7FS and algEL7RFLAG, algEL8FS and algEL8RFLAG, and algEL9FS and algEL9RFLAG). The plasmid template was removed by hydrolysis with DpnI. The two PCR products were mixed in equimolar amounts, and hybridization was achieved by incubation in H buffer (150 mM NaCl, 25 mM Tris, 20 mM EDTA, pH 8.0) at 99°C for 3 min, followed by three cycles of 65°C for 5 min and 30°C for 40 min. The resulting mixture was used to transform competent *E. coli* TOP 10 cells. Selection for cells coating the new plasmid was performed on ampicillin-containing medium, and the plasmids were extracted. Insertion of the 24-bp FLAG-encoding region was confirmed by DNA sequencing of the ORF, resulting in plasmids pGEMTEasy:*algE*L1FLAG to pGEMTEasy:*algE*L9FLAG. The *algE* (FLAG)-containing fragments were hydrolysed and inserted into pBBR1MCS-5, as described above, resulting in plasmids pBBRMCS-5:*algE*L1FLAG, pBBRMCS-5:*algE*L2FLAG, pBBRMCS-5:*algE*L3FLAG, pBBRMCS-5:*algE*L4FLAG, pBBRMCS-5:*algE*L5FLAG, pBBRMCS-5:*algE*L6FLAG, pBBRMCS-5:*algE*L7FLAG, pBBRMCS-5:*algE*L8FLAG, and pBBRMCS-5:*algE*L9FLAG.

Alginate production assays. Two millilitres of an overnight culture was harvested at 4°C and washed twice with saline. Then, 200 µl of the cell suspension was plated onto PIA medium and incubated 72 h at 37°C. Cells were scraped off of two agar plates by using a sterile spatula and were washed twice with 100 ml of saline (retaining the alginate-containing supernatant for subsequent precipitation). The cellular sediments were freeze-dried, and the final weight was determined. The alginate-containing supernatants were precipitated with 1 volume of ice-cold isopropanol, and the alginate was harvested and freeze-dried. For further purification, the precipitated alginate was redissolved in 0.05 M Tris-HCl-10 mM $MgCl_2$ (pH 7.4) to a final concentration of 0.5% (wt/vol), followed by incubation with 15 µg of DNase I/ml and 15 µg of RNase A/ml at 37°C for 6 h. Pronase E was added to a final concentration of 20 µg/ml, and this solution was incubated for a further 18 h at 37°C. The solutions were dialyzed against 5 litres of ultrapure H2O for 48 h. Alginate was precipitated with 1 volume of ice-cold isopropanol and freeze-dried for quantification and uronic acid analysis.

The levels of free uronic acids (alginate degradation products) in the supernatants of 2 ml of overnight cultures were measured. The total uronic acid content of the supernatant was determined as described below; the supernatants were filtered with Amicon Ultra-0.5 (Millipore) centrifugal filter devices (nominal molecular mass cutoff, 10 kDa), and the flowthrough was collected. The uronic acid content of the flowthrough (which contained free uronic acids and short-length alginate degradation products) was determined as described below.

Uronic acid assay. The alginate concentrations were assayed by the uronic acid assay described previously (12) by using alginic acid from brown seaweed (Sigma-Aldrich) as the standard. Briefly, the alginate samples were dissolved in 200 µl ultrapure H_2O at concentrations of between 0.25 and 0.05 mg/ml. The sample was mixed with 1.2 ml tetraborate solution (0.0125 M disodium tetraborate in concentrated sulfuric acid), and the mixture was incubated on ice for 10 min. The mixtures were incubated at 100°C for 5 min and then cooled down on ice for a further 5 min. Twenty microliters of *m*-hydroxybiphenyl reagent (0.15% *m*-hydroxybiphenyl in 0.125 M NaOH) was added and the reaction mixtures were mixed for 1 min. For each sample or dilution, a negative control was assayed by using 0.0125 M NaOH instead of the hydroxybiphenyl reagent. The uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

Purification of outer membranes. Strains of *P. aeruginosa* were grown overnight in LB medium containing the appropriate antibiotics. The cells were harvested by centrifugation (at 5,000 x *g* for 1 h) and washed twice with 1 volume of 10 mM HEPES (pH 7.4). The cells were placed in 15 ml 10 mM HEPES with Roche complete mini-EDTA-free protease inhibitor and sonicated on ice for 12 cycles of 15 s of sonication, followed by a 15-s cooldown. Cellular debris and the remaining intact cells were sedimented by centrifugation (at 5,000 x *g* for 1 h). The total membrane fraction was then isolated by centrifugation at 100,000 x *g* for 2 h. The supernatant (soluble fraction) was removed, the sediment was resuspended in 1 volume of 10 mM HEPES containing 0.7% (wt/vol) *N*-lauroylsarcosine, and the suspension was incubated at room temperature with shaking for 2 h to selectively solubilize the cytoplasmic membrane. This was then centrifuged at 100,000 x *g* for 2 h; the resulting sediment represents the outer membrane fraction. The total protein concentration of the respective fractions was determined by using a Quant-iT protein assay kit (Invitrogen).

Analysis of outer membrane proteins. Fifteen micrograms of total protein was then separated by SDS-PAGE on 8% polyacrylamide gels. The resulting gels were then either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane by using an iBlot dry blotting system (Invitrogen). The nitrocellulose membrane was blocked with 5% (wt/vol) skim milk powder in Tris-buffered saline with 0.1% Tween 20 for 1 h and was subsequently probed with 1 μg/ml anti-FLAG (M2) tag antibody conjugated to horseradish peroxidase (HRP; Abcam, Cambridge, United Kingdom). The membrane was washed, and the bound antibodies were resolved with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) and developed on X-ray film. Bands suspected of being AlgE or the AlgE mutants were identified by tryptic peptide fingerprinting by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS).

Results

Construction of an isogenic knockout mutant of *algE*. To investigate the requirement for AlgE in alginate biosynthesis, a marker-free *algE* deletion mutant of alginate-overproducing strain *P. aeruginosa* PDO300 was generated. This mutant showed a nonmucoid phenotype when it was grown on solid medium. The outer membrane protein profiles showed that AlgE was absent from PDO300Δ*algE* (Fig. 1, left panel). It has been shown that the lack of mucoidity associated with some deletion mutants (*algK*, *algG*, and *algX*) is distinct from the biosynthesis/polymerization of alginate. These mutants secrete alginate degradation products (free uronic acids), which have been shown to be the products of alginate lyase, AlgL (74-76, 138). To address this for the Δ*algE* mutant, the culture supernatants of the respective mutants were filtered and the uronic acid contents of the filtrates (containing alginate degradation products) were determined. Alginate degradation products could be detected in PDO300Δ*algE*(pBBR1MCS-5) at levels 9.7 times that of PDO300(pBBR1MCS-5) and 3.2 times that of the positive control, PDO300Δ*algX*(pBBR1MCS-5) (Table 2). Only small amounts of uronic acid were detected from the negative control, PDO300Δ*alg8*(pBBR1MCS-5) (Table 2). These results suggest that the Δ*algE* mutant is capable of the synthesis/polymerization of alginate but that it is subsequently degraded in the periplasm.

Complementation of the PDO300Δ*algE* isogenic knockout mutant. To verify that the observed loss of mucoidity was not due to polar effects on other genes in the alginate biosynthesis operon, a plasmid containing the *algE* ORF (pBBR1MCS-5:*algE*) was used to complement the mutant in *trans*. This plasmid was able to restore the mucoid phenotype as well as the production of alginate to levels beyond (five times greater) that of the parent strain containing the vector control PDO300(pBBR1MCS-5) (Table 2). This indicates that there were no polar effects in the deletion mutant and demonstrates the requirement of AlgE for the mucoid phenotype and the formation of intact and extracellular alginate. Interestingly, complemented strain PDO300Δ*algE*(pBBR1MCS-5:*algE*) produced more alginate degradation products than PDO300(pBBR1MCS-5) (53.6% and 8.4% total uronic acids, respectively) (Table 2). The presence of AlgE in the outer membrane was also restored (Fig. 1, left panel).

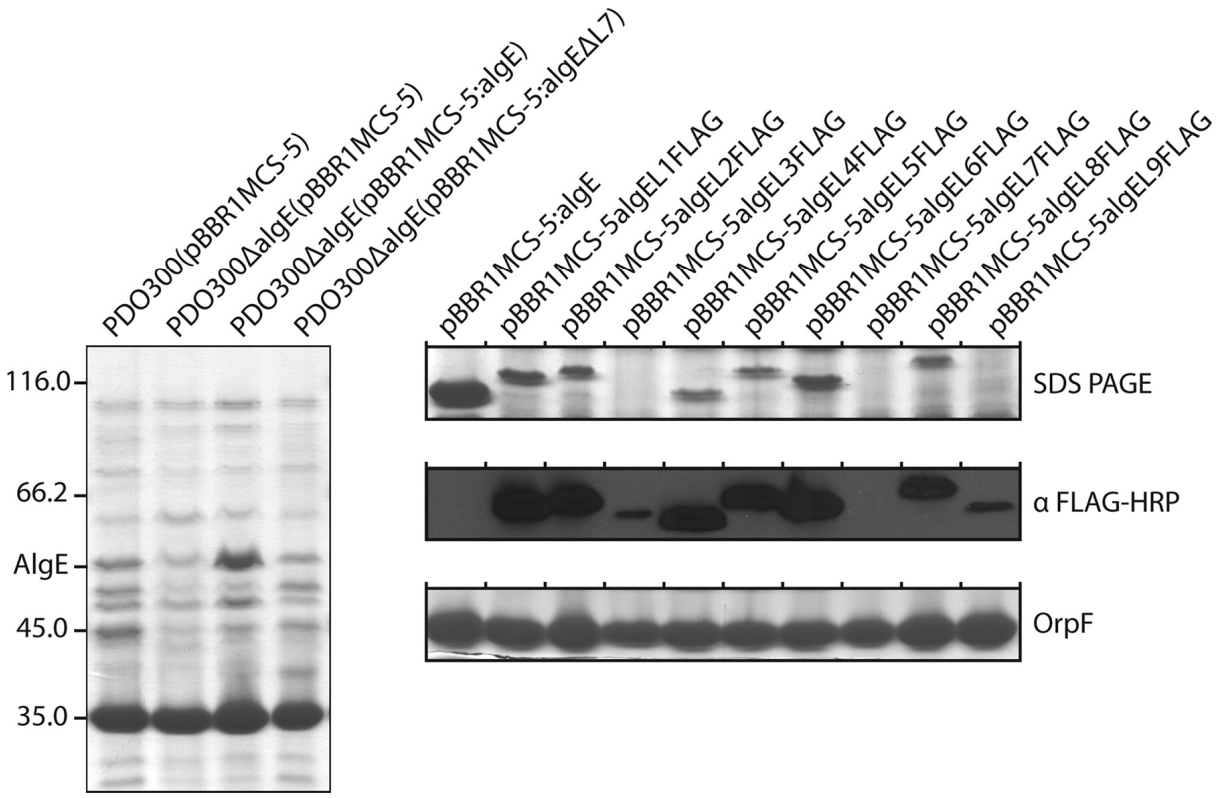

Figure 1. (Left panel) Outer membrane profiles of the Δ*algE* mutant and the Δ*algE* mutant harboring various plasmids showing the absence or presence of AlgE. Faint bands with the same apparent molecular mass (indicated on the left, in thousands) as AlgE present in the PDO300Δ*algE*(pBBR1MCS-5) and PDO300Δ*algE*(pBBR1MCS-5:*algE*ΔL7) outer membrane profiles were identified as flagellin type B protein by MALDI-TOF MS. (Right panels) Presence of tagged AlgE proteins in the outer membrane, as indicated by Western immunoblotting (middle). Constitutively produced OprF was used to standardize the samples (bottom).

Table 2: Production of alginate and free uronic acids by Δ*algE* mutants.

Strain	Alginate (g/g CDW) ± SD [a]	Free uronic acids (g/gCDW) ± SD	
PDO300(pBBR1MCS-5)	0.216 ± 0.027	Total [b]	0.524 ± 0.076
		Filtrate [c]	0.044 ± 0.009
PDO300Δ*algE*(pBBR1MCS-5)	ND[e]	Total	0.486 ± 0.050
		Filtrate	0.425 ± 0.039
PDO300Δ*algE*(pBBR1MCS-5:*algE*)	1.096 ± 0.251	Total	0.500 ± 0.014
		Filtrate	0.268 ± 0.010
PDO300Δ*algE*(pBBR1MCS-5:algEΔL7)	ND	Total	0.249 ± 0.007
		Filtrate	0.261 ± 0.031
PDO300Δ*algX*(pBBR1MCS-5)	ND	Total	0.138 ± 0.002
		Filtrate	0.131 ± 0.001
PDO300Δ*alg8*(pBBR1MCS-5)	ND	Total	0.012 ± 0.002
		Filtrate	0.013 ± 0.001

[a] SD - standard deviation
[b] Total - concentration of uronic acids in the unfiltered culture media (including alginate and free uronic acids)
[c] Filtrate - concentration of uronic acids in the filtered culture media (free uronic acids)
[e] ND – None Detected

Predicted topology of AlgE. The PRED-TMBB transmembrane β-strands prediction program for Gram-negative bacteria outer membrane proteins (5) predicts that the mature AlgE protein has 18 transmembrane β strands with nine extended surface-exposed loops and eight short periplasmic turns (Fig. 2). This is similar to what was predicted previously (126, 128). A search through the HHpred program (162) did not reveal homology to any annotated protein families or domains with known structures with which to refine this model.

The extracellular loop 7 region of AlgE is proposed to be required for the translocation of the alginate polymer. The proposed surface-exposed loop 7 is significantly longer than the other loops and contains a high density of positively charged amino acid residues which are conserved among all *Pseudomonas* species in the *Pseudomonas* Genome Database sequenced (178) (data not shown), as well as AlgJ from *Azotobacter vinelandii* (126). A conserved cluster of these positively charged residues is present at the apex of the loop region, suggesting that this region may be folded into the pore, facilitating the translocation of the anionic alginate chain through the pore. A vector encoding AlgE with an 18-amino-acid deletion in this conserved region at the apex of loop 7 (pBBR1MCS-5:*algE*ΔL7) was constructed in an attempt to analyse any role that this region may have in alginate translocation and/or protein folding/structure. This plasmid could not restore alginate production in the Δ*algE* mutant, nor could the AlgE variant be localized to the outer membrane (Table 2; Fig. 1). This strain produced free uronic acids, but at levels 1.6 times lower than those for the *algE* deletion mutant, PDO300Δ*algE*(pBBR1MCS-5).

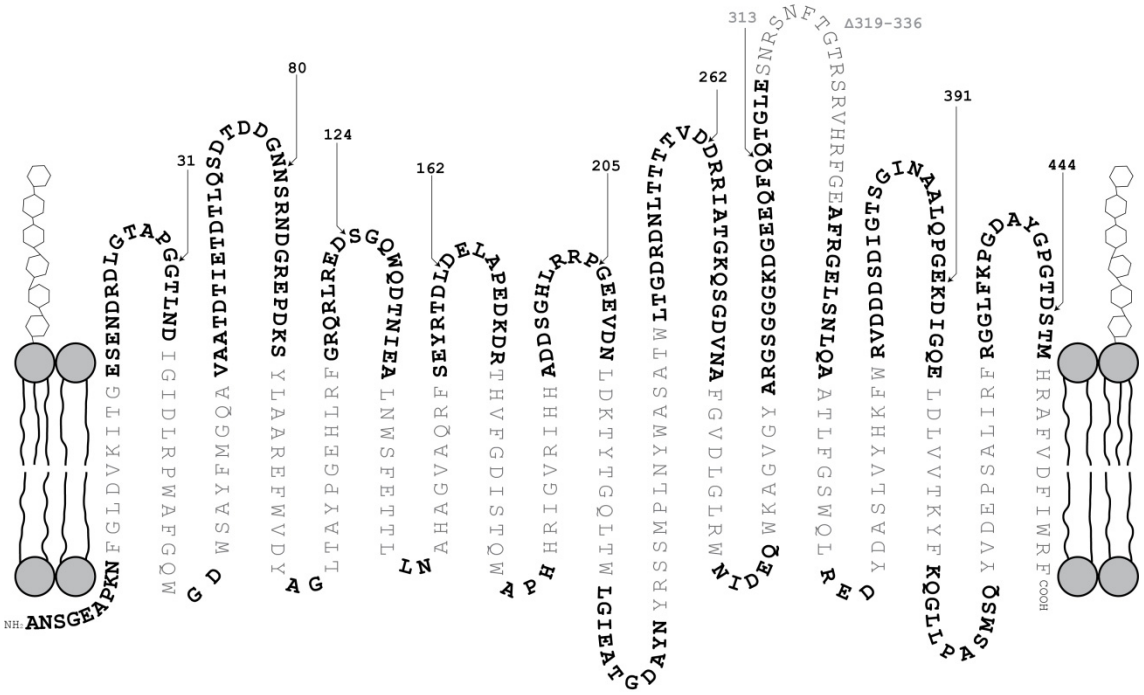

Figure 2. Predicted topology of the outer membrane β-barrel AlgE. Predicted transmembrane β sheets are indicated in regular text (nonbold), extracellular loops at the top are indicated in boldface, and periplasmic turns at the bottom are indicated in bold. Locations where the FLAG epitopes were inserted are indicated by triangles, and the positions (relative to the position of the cleavage of the signal peptide) are indicated; black numbers, the insertion was permissive, i.e., alginate production was restored; gray numbers, the insertion was nonpermissive. The deleted region of loop 7 is indicated by regular gray text (nonbold).

AlgE membrane topology analysis. To test the predicted AlgE model presented in Fig. 2, site-directed epitope insertion mutagenesis targeting the proposed extracellular loops was done. This method utilizes the observation that insertions in the hydrophilic loop regions distant from the β-barrel core are usually tolerated, permitting the correct formation of the β barrel required for insertion of the protein into the outer membrane (112, 131). The FLAG epitope was inserted into the predicted loop regions for loops 1 to 9 by SLIM (26) at the positions indicated in Fig. 2. Plasmids encoding these tagged proteins were introduced into the Δ*algE* mutant strain, the outer membrane protein profiles were obtained by SDS-PAGE, and the respective proteins were subjected to Western immunoblotting with anti-FLAG HRP-conjugated antibody. Insertion of the FLAG epitope into loops 1 to 6, 8, and 9 were tolerated, and the protein could be detected in the respective outer membranes. Interestingly, although the tagged proteins have identical deduced molecular masses of 52.2 kDa, they migrated significantly differently by SDS-PAGE: insertions in loops 1, 6, and 9 at 53.5 kDa; loop 2 at 54.7 kDa; loop 3 at 52.3 kDa; loop 4 at 51.4 kDa; loop 5 at 55 kDa; and loop 8 at 56.5 kDa. Additionally, AlgEL3FLAG and AlgEL9FLAG appeared to be present at significantly smaller quantities than the other AlgE

proteins (Fig. 1, right panels). AlgE variants harbouring insertions in loops 1 to 6, 8, and 9 restored alginate production in the Δ*algE* mutant (Table 3), indicating that the core protein structure was not disrupted and that no functional regions essential for alginate formation were affected. Surprisingly, insertion of the epitope into the proposed loop 7 region was not tolerated. This protein could not be detected in the outer membrane and did not restore alginate formation (Table 3).

Discussions

The role of AlgE in alginate production and its membrane topology were investigated in the present study. The isogenic marker-free *algE* deletion mutant was nonmucoid and did not produce alginate (Table 2). Alginate secretion could be restored by introduction of only the *algE* gene in *trans*. This indicates that AlgE is essential for the mucoid phenotype. The *algE* deletion mutant secreted free uronic acids, indicating that alginate is still being polymerized but is presumably degraded by AlgL in the periplasm (Table 2). Thus, like AlgK, AlgX, AlgG, and AlgL, AlgE is not required for the polymerization or translocation of alginate across the inner membrane but is required for the successful translocation of alginate through the periplasm and across the outer membrane (2, 57, 74-76, 138). This supports the hypothesis that alginate is guided through the periplasm by a multi-protein complex or scaffold containing periplasmic proteins AlgK, AlgX, AlgG, and AlgL and outer membrane protein AlgE. The loss of any of these proteins results in the lack of the integrity of this complex and degradation of the alginate chain. It is possible that the predicted protein scaffold is attached to the outer membrane via AlgE, perhaps via the tetratricopeptide repeat motif of AlgK often found in adaptor proteins involved in the assembly of multi-protein complexes and/or the membrane fusion domain of Alg44 (11, 135). Indeed, mutants lacking Alg44 show reduced levels of AlgE in the outer membrane (114).

The in *trans* complementation of the Δ*algE* mutant still resulted in the secretion of free uronic acids (albeit less than the level of secretion by the Δ*algE* mutant) (Table 2). Since AlgE is overproduced in the outer membrane (Fig. 1) of the complemented Δ*algE* mutant, it could interfere with the stoichiometry of proteins in the proposed scaffold complex. Hence, the presence of an increased AlgE copy number might, to a certain extent, increase the number of functional multi-protein complexes contributing to alginate overproduction, while the additional copy number might also lead to dysfunctional multi-protein complex formation, causing the degradation of some of the alginate as it traverses the periplasm.

Deletion of the conserved extracellular loop 7 region likely interfered with the folding pathway of AlgE, as evidenced by its absence in the outer membrane and its inability to restore alginate production (Fig. 1; Table 3). This suggests that the deleted region is essential for the functional folding of AlgE. The deleted region contains a relatively high concentration of conserved (among all sequences of *Pseudomonas* spp. in the *Pseudomonas* Genome Database (178) as well as AlgJ of *Azotobacter vinelandii*) positively charged amino

acid residues, and therefore, it was proposed that this region is involved in the active translocation of the anionic alginate chain through the outer membrane (Fig. 2). Alternatively, it cannot be ruled out that the topology prediction could be incorrect and that the deletion site may be in an unpredicted transmembrane region.

Site-directed epitope insertion mutagenesis of all predicted extracellular loop regions was completed to probe the predicted membrane topology of AlgE. Similar methods have been utilized to probe the topologies of various outer membrane proteins (112, 131, 175, 176). As expected, insertion of the FLAG epitope into eight of nine loop regions was tolerated and did not disrupt the β-barrel structure, allowing insertion into the outer membrane (Fig. 1). These proteins were also capable of complementing the Δ*algE* mutant by restoring alginate production (Table 3). Hence, supporting evidence that these tagged regions are loop regions not involved in the formation of the β barrel through which alginate is exported was provided. When the FLAG epitope was inserted into the predicted loop 7 region, it was not tolerated, even though this insertion site is the most distant from any predicted β sheets among the loop insertions. However, it was consistent with the finding that the deletion of an 18-amino-acid region 5 amino acid residues upstream of the insertion site in this loop was not tolerated (Fig. 1; Table 3). Loop 7, which was proposed to be actively involved in the transport of alginate, might be folded and might bend into the hydrophobic β-barrel core, similar to the "eyelet" region described in other porins. Indeed, this loop 7 contains a concentration of charged residues similar to that found in other eyelet loops (13, 77, 165, 173, 174). Consequently, insertion of the highly hydrophilic FLAG tag into this loop could disrupt the hydrophobic core of the barrel and prevent folding.

Under the conditions used in this study, AlgE did not migrate aberrantly as a 54-kDa protein, as described elsewhere (29, 128), but migrated at its deduced molecular mass of 51.2 kDa. Additionally, even though they had identical deduced molecular masses, the epitope-tagged AlgE variants migrated differently, an observation similar to that reported previously for OprH (131) (Fig. 1). This can be explained by the observation that outer membrane proteins are known for their extraordinary stability and have been reported to exhibit heat-modifiable characteristics, resulting in differences in their migration patterns when they are subjected to SDS-PAGE (59).

In conclusion, experimental evidence was provided for the requirement of AlgE for the secretion of alginate, whereas it is not required for alginate polymerization and translocation into the periplasm. Furthermore, it was demonstrated that loop 7 is required

for the functional folding of AlgE, while the other insertion mutants support the proposed membrane topology as a β barrel with 18 β strands and 9 loops. Overall, the data suggest that AlgE not only might be involved in the secretion of alginate but also contributes to the proposed protein scaffold, guiding and protecting the nascent alginate chain through the periplasm and outer membrane.

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Supplementary Table 1

Oligonucleotides		
algE1N-Ec5	GCAGGATATCGTGAAGATCACCGGCGAATCG	This study
algE1C-Ba	TGTAGGATCCGTCGAGGTCGGTGCGGTATTC	This study
algE2N-Ba	AGAGGGATCCGGAGAGCAATCGCTCCAACTTCAC	This study
algE2C-Ec5	GTCCGATATCCAGAAGCGCCAGATGAAGTCGAC	This study
algEup	CAATTGCTCAAGCGCGAACAACAG	This study
algEdown	GACCGGCTGCGGTTTCTCCACC	This study
algEN(HiSDNd)	ACCAAAGCTTAGGAGAGAAAGCATATGAACAGCTCCCGTTCCGTCA ACC	This study
algEC(Ba)	GACGGATCCTCAGAAGCGCCAGATGAAGTCGAC	This study
algEDelL7N(Ba)	TACAAGGATCCCTCCAGCCCGGTCTGCTGGAACTGCTC	This study
algEDelL7C(Ba)	AGCTAGGATCCTTCCGCGGCGAACTGAGCAACCTCCAG	This study
algEC(SaI)	GACGAGCTCTCAGAAGCGCCAGATGAAGTCGAC	This study
algEL1F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**GGCACCCTCAACGACATCGG	This study
algEL1R$_{S}$	GCCGGGAGCGGTGCCGAGGT	This study
algEL1F$_{S}$	GGCACCCTCAACGACATCGG	This study
algEL1R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GCCGGGAGCGGTGCCGAGGT	This study
algEL2F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**AACAGCCGCAACGACGGTCG	This study
algEL2R$_{S}$	GTTGCCGTCGTCGGTGTCCG	This study
algEL2F$_{S}$	AACAGCCGCAACGACGGTCG	This study
algEL2R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GTTGCCGTCGTCGGTGTCCG	This study
algEL3F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**AGCGGCCAGTGGCAGGACAC	This study
algEL3R$_{S}$	GTCTTCCCGCAGGCGCTGGC	This study
algEL3F$_{S}$	AGCGGCCAGTGGCAGGACAC	This study
algEL3R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GTCTTCCCGCAGGCGCTGGC	This study
algEL4F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**GACGAACTGGCTCCGGAGGA	This study
algEL4R$_{S}$	GAGGTCGGTGCGGTATTCGC	This study
algEL4F$_{S}$	GACGAACTGGCTCCGGAGGA	This study
algEL4R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GAGGTCGGTGCGGTATTCGC	This study
algEL5F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**GGCGAGGAAGTCGACAACCT	This study
algEL5R$_{S}$	GGGGCGGCGCAGGTGGCCGC	This study
algEL5F$_{S}$	GGCGAGGAAGTCGACAACCT	This study
algEL5R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GGGGCGGCGCAGGTGGCCGC	This study
algEL6F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**GACCGGCGCATCGCCACCGG	This study
algEL6R$_{S}$	GTCGACCGTGGTGGTGGTCA	This study
algEL6F$_{S}$	GACCGGCGCATCGCCACCGG	This study
algEL6R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GTCGACCGTGGTGGTGGTCA	This study
algEL7F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**CAGACCGGGCTGGAGAGCAAT	This study
algEL7R$_{S}$	CTGGAACTGCTCCTCGCCGTC	This study
algEL7F$_{S}$	CAGACCGGGCTGGAGAGCAAT	This study
algEL7R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**CTGGAACTGCTCCTCGCCGTC	This study
algEL8F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**AAGGACATCGGCCAGGAACT	This study
algEL8R$_{S}$	CTCGCCCGGTTGCAGGGCGG	This study
algEL8F$_{S}$	AAGGACATCGGCCAGGAACT	This study
algEL8R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**CTCGCCCGGTTGCAGGGCGG	This study
algEL9F$_{FLAG}$	**GACTACAAGGACGACGACGACAAG**TCGACCATGCACCGCGCCTT	This study
algEL9R$_{S}$	GTCGGTGCCCGGCCCGTAGG	This study
algEL9F$_{S}$	TCGACCATGCACCGCGCCTT	This study
algEL9R$_{FLAG}$	**CTTGTCGTCGTCGTCCTTGTAGTC**GTCGGTGCCCGGCCCGTAGG	This study

Chapter IV

MucR, a membrane anchored diguanylate cyclase involved in alginate biosynthesis by *Pseudomonas aeruginosa*

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Abstract

Alginate biosynthesis by *Pseudomonas aeruginosa* was shown to be regulated by the intracellular second messenger bis-(3'-5')-cyclic-dimeric-GMP (c-di-GMP), and binding of c-di-GMP to the membrane protein Alg44 was required for alginate production. In this study, PA1727, a c-di-GMP-synthesizing enzyme was functionally analyzed and identified to be involved in regulation of alginate production. Deletion of the PA1727 gene in the mucoid alginate-overproducing *P. aeruginosa* strain PDO300 resulted in a nonmucoid phenotype and an about 38-fold decrease in alginate production; thus, this gene is designated *mucR*. The mucoid alginate-overproducing phenotype was restored by introducing the *mucR* gene into the isogenic Δ*mucR* mutant. Moreover, transfer of the MucR-encoding plasmid into strain PDO300 led to an about sevenfold increase in alginate production, wrinkly colony morphology, increased pellicle formation, auto-aggregation, and the formation of highly structured biofilms as well as the inhibition of swarming motility. Outer membrane protein profile analysis showed that overproduction of MucR mediates a strong reduction in the copy number of FliC (flagellin), required for flagellum-mediated motility. Translational reporter enzyme fusions with LacZ and PhoA suggested that MucR is located in the cytoplasmic membrane with a cytosolic C terminus. Deletion of the proposed C-terminal GGDEF domain abolished MucR function. MucR was purified and identified using tryptic peptide fingerprinting and matrix-assisted laser desorption ionization-time of flight mass spectrometry. Overall, experimental evidence was provided suggesting that MucR specifically regulates alginate biosynthesis by activation of alginate production through generation of a localized c-di-GMP pool in the vicinity of Alg44.

Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen which is responsible for systemic infection of immunocompromised patients and severe chronic infections of the lungs of cystic fibrosis (CF) patients (31, 32). Establishment of a chronic CF lung infection coincides with production of copious amounts of the exopolysaccharide alginate leading to the formation of persistent biofilms which prevent the diffusion of antibiotics and protect cells from the host immune response (31, 92). Early after the onset of infection, *P. aeruginosa* switches to a genetically stable alginate-overproducing mucoid variant capable of forming persistent biofilms (98, 99, 130). Alginates are linear unbranched exopolysaccharides consisting of β-1,4-linked monomers of β-D-mannuronic acid and its C5-epimer β-L-guluronic acid (132, 136). The switch to mucoidity and the complex transcriptional regulation of alginate biosynthesis have extensively been investigated (17).

Recently it has become apparent that an additional posttranscriptional level of regulation is playing a role in alginate biosynthesis in *P. aeruginosa*. The membrane-anchored alginate biosynthesis protein Alg44, which is essential for alginate production, contains a bis-(3'-5')-cyclic-dimeric-GMP (c-di-GMP) binding/sensing PilZ domain in its C terminus (135). This PilZ domain was demonstrated to bind c-di-GMP and was essential for alginate biosynthesis (3, 102).

The secondary messenger c-di-GMP is a central regulator of bacterial physiology and has been linked to diverse physiological responses, such as exopolysaccharide production, motility, biofilm formation, and the production of adhesive surface organelles (139). *P. aeruginosa* PAO1 contains at least 38 genes encoding proteins shown to be involved in the production (diguanylate cyclases [DGC]) and/or the breakdown (phosphodiesterases [PDE]) of c-di-GMP (88). Sequence analysis of these enzymes indicated the presence of domains involved in signal transduction, which suggested that these proteins function in response to diverse signals. It was also suggested that localized c-di-GMP pools might exist inside the cell, enabling spatially resolved regulation of physiological responses (88). At present it is unclear how or if any of these different proteins influence alginate biosynthesis. Since the c-di-GMP-binding Alg44 protein is localized in the cytoplasmic membrane, presumably as a subunit of the alginate polymerization/secretion multi-protein complex, membrane-anchored DGCs could provide localized pools of c-di-GMP for activation of alginate production. Putative membrane-anchored PA1727 (MucR) comprises a DGC (GGDEF) as well as a PDE (EAL) and a conserved integral membrane-sensing MHYT domain, which was proposed to sense oxygen, CO, or NO (51). DGC activity

but not PDE activity has previously been demonstrated (88). Oxygen and NO have previously been shown to influence alginate production (21, 46). In this study, MucR was assessed with respect to its involvement in regulation of alginate production.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. All *Escherichia coli* strains were grown in LB medium at 37°C, and *E. coli* S17-1 (158) was used for conjugative transfer of mob site-containing pBBR1MCS-5 (85) derivatives. When required, antibiotics were added to the media at the following concentrations: ampicillin, 100 μg/ml; and gentamicin, 10 μg/ml. *P. aeruginosa* was cultivated in LB or *Pseudomonas* isolation agar (PIA) medium at 37°C, and, if required, gentamicin was added at a concentration of 100 μg/ml.

Isolation, analysis, and manipulation of DNA. General cloning procedures were performed as described previously (146). Deoxynucleoside triphosphate, *Taq*, and Platinum *Pfx* polymerases were purchased from Invitrogen. DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic sequencer.

Table 1. Bacterial strains, plasmids and Oligonucleotides used in this study

Strain, Plasmid, or oligonucleotide	Description or sequence	Source or reference
P. aeruginosa		
PAO1	Prototrophic wild-type strain; Alg^-	(72)
PDO300	*muc22A* isogenic mutant derived from PAO1	(99)
PAO1ΔPA1727	Isogenic *mucR* (PA1727) deletion mutant derived from PAO1	This study
PDO300ΔPA1727	Isogenic *mucR* (PA1727) deletion mutant derived from PDO300	This study
PDO300Δ*alg8*	Isogenic *alg8* deletion mutant derived from PDO300. Cannot produce alginate.	(137)
PAO1Δ*pslA*	Isogenic *pslA* deletion mutant derived from PDO300. Does not produce the Psl polysaccharide.	(116)
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi-1 proA hsdR17* (r_K^- m_K^+) *recA1*; *tra* gene of plasmid RP4 integrated in chromosome	(154)
Plasmid		
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	(85)
pBBR1MCS-5:*mucR*	*Kpn*I-*Cla*I fragment comprising *mucR* inserted in to vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*his	Translational MucR-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*lacZ	Translational MucR-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-	Translational MucR-PhoA fusion, inserted into	This study

5:*mucR*phoA	vector pBBR1MCS-5	
pBBR1MCS-5:*mucR*(277)lacZ	Truncated translational MucR-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucR*(277)phoA	Truncated translational MucR-PhoA fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*wspR*	*Xba*I-*Sac*I fragment comprising *wspR* inserted in to vector pBBR1MCS-5	This study
pBBR1MCS-5:*rocR*	*Xba*I-*Sac*I fragment comprising *rocR* inserted in to vector pBBR1MCS-5	This study
pEX100T	Ap^r Cb^r, gene replacement vector containing *sacB* gene for counterselection	(69)
pEX100TΔ*mucR*Gm	Ap^r Cb^r Gm^r; vector pEX100T with SmaI-inserted *mucR* deletion construct	This study
pPS865	Ap^r Gm^r; source of 1,100-bp BamHI fragment comprising *aacC1* gene flanked by FRT signal sequences	(69)
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(69)
pPHO7	Ap^r; *phoA* without signal sequence	(58)
pJE608	LacZ lacking the first 8 amino acids with promoter P*tac* in pMMB67EH	(40)
Primers		
PA1727C1-Bbr	TATACCACGTGCTGGCGAGCAACTGCTCGGGCATCGGCCTG	
PA1727C2-Ba	TCAATGGATCCGCAGATCGGCGAGAGGGTGCTCGACGAAGC	
PA1727N1-Ba	AAGTGGGATCCGATCACCGCGATCAGGATGGAGAGG	
PA1727N2-Brb	CTGCTCACGTGCAGGTTCTTGTCGCCTTTTCCCTGATTGTGG	
PA1727C(Cla)	GAGTAATCGATAAATCAGGCGACGATGGCGAGCAACTGCTCG	
PA1727N(KpSDNd)	AGCAAGGTACCAGGAGACGCTCATATGCTTATCAGCAGCTACACCCAGGTTATT	
PA1727C-tga(BamHI-ClaI)	GAGTAATCGATAAATCAAGGATCCGCGGCGACGCTGGCGAGCAACTGCTGCGCCGGCATCGG	
PA1727(277)Fus(BamHI-ClaI)	GAGTAATCGATAAATCAAGGATCCGCGAGGAGCATGCGGTTGGGCAGCTTGGTCAGGTTGTCGTGCAGG	
PA3702N(XbSDNd)	GCGTCGTCTAGAAGGAGAGAGACATATGCACAACCCTCATGAGAGCAAGACCGACC	
PA3702C(SacI)	GGCTGGAGCTCAAATCAGCCCGCCGGGGCCGGCGGCACC	
PA3947N(XbSDNd)	GCGTCGTCTAGAAGGAGGGACCCATATGAATGATTTGAATGTTCTGGTGTTGGAGG	
PA3947C(SacI)	GGCTGGAGCTCAAATCAGGATCCGGAGCAATAGTCGAGAAAGTGC	
LumioHis(BaXbdirect)	GATCCATGTTGTCCTGGCTGTTGCGGTGGCGGCACCGGTCATCATCACCATCACCATTGAT	
LumioHis(BaXbcomplement)	CTAGATCAATGGTGATGGTGATGATGACCGGTGCCGCCACCGCAACAGCCAGGACAACATG	

Construction of *mucR* deletion mutant. Two regions of the *mucR* gene were amplified by using *Taq* polymerase with primers PA17271N2-Bbr, PA17271N1-Ba, PA17272C2-Ba, and PA17272C1-Bbr. Region PA1727N (432 bp) comprised bases 21 to 452, and region PA1727C (555 bp) comprised bases 1493 to 2047, relative to the designated *mucR* coding region (164). Both PCR products were hydrolysed with BamHI, ligated together, and inserted into vector pGEM-TEasy (Promega). Vector pPS856 (69) was hydrolysed with BamHI, releasing an about 1,100-bp fragment containing the *aacC1* gene (encoding gentamicin acetyltransferase) flanked by two Flp recombinase target sites. The 1,100-bp BamHI fragment (*aacC1* gene) was inserted into the BamHI site of plasmid pGEM-TEasy:Δ*mucR*NC, resulting in plasmid pGEM-TEasy:Δ*mucR*Gm. The Δ*mucR*Gm-comprising DNA fragment was excised using restriction endonuclease BbrP1, and the corresponding 2,087-bp fragment was inserted into the SmaI site of vector pEX100T (69, 154), resulting in plasmid pEX100TΔ*mucR*Gm. *E. coli* S17-1 was used as a donor for the transfer of plasmid pEX100TΔ*mucR*Gm into *P. aeruginosa* strains, and transconjugants were selected on mineral salt medium (150) containing 300 μg of gentamicin/ml and 5% (wt/vol) sucrose. Cells growing on this selective medium should have emerged from double-crossover events. Gene replacement was confirmed after the subculture of cells on PIA medium containing 300 μg of gentamicin/ml and using PCR with primers PA1727up and PA1727down. *E. coli* S17-1 was used to transfer the Flp recombinase-encoding vector pFLP2 (69) into *P. aeruginosa* Δ*mucR*Gm strains, and after 24 h of cultivation on PIA medium containing 5% (wt/vol) sucrose, gentamicin- and carbenicillin-sensitive cells were analysed by PCR for loss of the gentamicin resistance cassette.

Complementation of the Δ*mucR* mutant. The *mucR* gene of *P. aeruginosa* PAO1 was amplified by PCR using the primers PA1727N(KpSDNd) and PA1727C(Cla). The PCR product was hydrolysed with KpnI and ClaI and was inserted into the KpnI and ClaI sites of the broad-host-range vector pBBR1MCS-5, resulting in plasmid pBBR1MCS-5:*mucR*. In addition a C-terminally hexahistidine-tagged MucR-encoding plasmid was constructed by using primers PA1727N(KpSDNd) and PA1727C-tga(BamHI-ClaI), and the resulting PCR product was inserted into pBBR1MCS-5 as described above. The resulting plasmid was then hydrolysed with BamHI and XbaI; a hexahistidine-encoding sequence was ligated into this using the complementary primer dimer pair LumioHis(BaXbdirect) and LumioHis(BaXbcomplement), resulting in plasmid pBBR1MCS-5:*mucR*His. In addition two plasmids carrying two other genes known to influence c-di-GMP levels were constructed. The genes *wspR* (PA3702) (encoding a highly active DGC) and PA3947 (encoding a highly active PDE) were amplified from the *P. aeruginosa* PAO1 genome using the 5'-end primers

PA3702N(XbSDNd) and PA3947N(XbSDNd) and the 3'-end primers PA3702C(SacI) and PA3947C(SacI), respectively. These PCR products were then hydrolysed with XbaI and SacI and inserted into the respective sites of pBBR1MCS-5, resulting in plasmids pBBR1MCS-5:PA3702 and pBBR1MCS-5:PA3947, respectively.

Construction of plasmids encoding reporter enzyme fusion proteins. For the generation of a plasmid encoding full-length MucR fused either to β-galactosidase (LacZ) or alkaline phosphatase (PhoA), the 3' end of *mucR* (*mucR* minus the stop codon) was amplified by PCR using the 5'-end primer PA1727N(KpSDNd) and the 3'-end primer PA1727C-tga(BamHI-ClaI). The PCR product was hydrolysed with KpnI and BamHI and was inserted into the respective sites of broad-host-range vector pBBR1MCS-5, resulting in plasmid pBBR1MCS-5:*mucR*(Δstop). XbaI-BamHI fragments of vectors pPHO7 (40) and pJE608 (56) were inserted into XbaI/BamHI-hydrolysed pBBR1MCS-5:*mucR*(Δstop) to construct translational LacZ (pBBR1MCS-5:*mucR*LacZ) and PhoA (pBBR1MCS-5:*mucR*PhoA) fusions, respectively. In addition, a translational fusion was constructed that encodes the first 277 amino acids of MucR fused to either LacZ or PhoA. The 3' region was amplified by using the 5'-end primer PA1727N(KpSDNd) and the 3'-end primer PA1727277(BamHI-ClaI). Fusion protein-encoding plasmids pBBR1MCS-5:*mucR*277LacZ and pBBR1MCS-5:*mucR*277PhoA were constructed as described above.

Subcellular localization of MucR. Strains of *P. aeruginosa* were grown overnight in LB medium containing the appropriate antibiotics. The cells were harvested by centrifugation (1 h at 5,000 x *g*) and washed with 1 volume of 10 mM HEPES (pH 7.4). Cells were placed in 15 ml 10 mM HEPES with Roche Complete Mini EDTA-free protease inhibitor, sonicated on ice for 12 cycles of 15-s sonication, and then cooled for 15 s. Cellular debris and the remaining intact cells were sedimented by centrifugation (1 h at 5,000 x *g*). The supernatant was centrifuged at 100,000 x *g* for 2 h. The supernatant (soluble fraction) was removed, and the sediment was washed in 1 volume of 10 mM HEPES and centrifuged under the same conditions. The resulting sediment represents the envelope fraction, which was dissolved in 10 mM HEPES. The total protein concentration of the respective fractions was determined using the Bradford method (20).

Outer membrane protein analysis. Cells were fractionated as described above. The cytosolic membrane fraction was then selectively solubilized from the total envelope fraction using 0.7% (wt/vol) *n*-lauroylsarcosine. The insoluble (outer membrane) fraction of the envelope fraction was then obtained by centrifugation at 100,000 x *g* for 1 h. Resulting outer membrane proteins were then separated via sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) and identified by tryptic peptide fingerprinting using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

β-Galactosidase and alkaline phosphatase activity assays. β-Galactosidase and alkaline phosphatase enzymatic assays were performed according to the methods by Miller (105) and Manoil (96), respectively. Between 2 and 20 μl of the various subcellular fractions was added to the reaction mixtures, and the specific activity was determined in U/mg total protein, with 1 U corresponding to the hydrolysis of 1 μmol of substrate (*o*-nitrophenyl-β-galactoside and *p*-nitrophenylphosphate for alkaline phosphatase and β-galactosidase, respectively) per 1 min at 37°C. The results are given as average values of at least four independent experiments.

Uronic acid assays. Alginate concentrations were assayed by a modification of the Blumenkrantz and Asboe-Hansen protocol (12), using purified *P. aeruginosa* PDO300 alginate (100% [wt/wt] uronic acid content) as a standard, as previously described (135). The uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

Swarming motility. Swarming motility was assessed by the method of Tremblay et al. (171). Briefly, plates consisted of modified M9 medium (20 mM NH_4Cl; 12 mM Na_2HPO_4; 8.6 mM NaCl; 1 mM $MgSO_4$; 1 mM $CaCl_2{\cdot}2H_2O$; and 10 mM dextrose, supplemented with 0.5% [wt/vol] Casamino Acids [Difco]) solidified with 0.5% (wt/vol) Bacto agar (Difco). Autoclaved medium was poured in petri dishes and dried under laminar flow for 60 min. Then swarm plates were immediately inoculated with 5 μl of stationary-phase bacterial culture and incubated at 30°C for 16 h.

Solid surface attachment assay. Attachment to a solid surface was assessed by a modified method described by Merritt et al. (104). Briefly, relevant strains were grown to saturation in LB medium. A total of 100 μl of these cultures was transferred to 4 wells of a sterile 96-well microtiter plate and incubated at 37°C for 1 h. Planktonic/nonadherent bacteria were removed by inverting the plate, and the plates were washed with water. A total of 125 μl of 0.1% crystal violet was added to each well, incubated at room temperature for 10 min, subsequently washed twice as described above, and allowed to air dry. Bound crystal violet was solubilized with the addition of 200 μl of 100% dimethyl sulfoxide. Absorbance was measured at 595 nm.

Continuous-culture flow cell biofilms. For biofilm analysis, *P. aeruginosa* strains were grown in continuous-culture flow cells (channel dimensions of 4 mm by 40 mm by 1.5 mm) at 37°C as previously described (22). Channels were inoculated with 0.5 ml of early-stationary-phase cultures containing approximately 2 x 109 cells ml–1 and incubated without flow for 4 h at room temperature. Flow was then started with a mean flow of 0.3 ml min–1, corresponding to a laminar flow with a Reynolds number of 5. The flow cells were then incubated at 37°C for 20 h. Biofilms were stained using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) and visualized first using phase-contrast microscopy and then confocal laser scanning microscopy (Leica SP5 DM6000B).

Detection and purification of MucR. The envelope fraction of the PDO300Δ*mucR* strain harbouring plasmid pBBR1MCS-5:*mucR*his was solubilized by the addition of 1% (vol/vol) Triton X-100 and incubated at room temperature for 2 h. The solubilized envelope fraction was subjected to affinity purification using Ni-NTA agarose (Qiagen). Briefly, the solubilized envelope fraction from 1 liter of a stationary-phase culture was resuspended in 20 ml of buffer A (50 mM NaH2PO4, 300 mM NaCl, 1% Triton X-100, pH 8.0) containing 10 mM imidazole, and 1 ml of a 50% (wt/vol) Ni-NTA agarose (Qiagen) was added and incubated with shaking at 4°C for 1 h. The Ni-NTA agarose was then loaded onto a gravity flow column, and the agarose was washed four times with 5 ml of buffer A containing 20 mM imidazole and four times with 5 ml of buffer A containing 50 mM imidazole. The bound proteins were eluted four times with 500 μl of buffer A containing 250 mM imidazole. Fractions were then subjected to SDS-PAGE analysis and immunoblotting analysis. In order to identify proteins, protein bands of interest were cut off the gel. Proteins were subjected to tryptic peptide fingerprinting. Identification of tryptic peptides was performed by collision-induced dissociation tandem mass spectrometry and enabled identification of proteins. For immunoblotting, proteins were transferred to a nitrocellulose membrane and hexahistidine-tagged proteins were detected using a SuperSignal HisProbe-HRP kit (Pierce).

Results

Primary structure analysis of MucR. The MucR (PA1727) sequence was attained from the complete *P. aeruginosa* PAO1 genome sequence (164). TMHMM (86) predicts that MucR has seven transmembrane regions, all located in the N-terminal 240 residues of the protein, with the N terminus residing in the periplasm and the C terminus residing in the cytosol. SMART (90) and Pfam (42) predicted an MHYT domain (51) in the second to seventh transmembrane regions. This domain consists of six transmembrane segments connected by short arginine-rich cytosolic loops. The second, fourth, and sixth transmembrane segments in the domain have a highly conserved amino acid motif, MHYTXM, located near the outer side of the inner membrane. It has been suggested that the MHYT domain serves as a sensing domain. The C terminus of MucR is predicted to contain a GGDEF domain, common to DGCs, at amino acids 252 to 423 and an EAL domain, conserved among PDE, at amino acids 433 to 679. A sequence database search (BLASTP) revealed the strongest similarity of 73% identity with the hypothetical protein ZP_00417207 from *Azotobacter vinelandii* belonging to one of the two bacterial genera (*Pseudomonas* and *Azotobacter*) comprising species capable of alginate production.

The Δ*mucR* mutant is defective in alginate biosynthesis. Deletion of 1,041 bp of the *mucR* gene in the alginate-overproducing *P. aeruginosa* strain PDO300 resulted in a nonmucoid colony phenotype on solid medium (Fig. 1). Interestingly, strains PDO300 and PAO1, each harbouring plasmid pBBR1MCS-5:*mucR* and thus additional *mucR* gene copies, showed small wrinkly colonies, while PDO300 was additionally surrounded by copious amounts of transparent extracellular alginate (Fig. 1). Alginate is the primary exopolymeric substance responsible for the mucoid phenotype, which suggested that MucR plays a role in biosynthesis or regulation of alginate biosynthesis in *P. aeruginosa*. To assess whether the Δ*mucR* mutant of strain PDO300 is still able to produce alginate, the extracellular polysaccharide was purified and the uronic acid (alginate) content was determined. The Δ*mucR* mutation caused a reduction in alginate production to nearly undetectable levels (Table 2). To confirm this was not due to polar effects, the *mucR* gene (pBBR1MCS-5:*mucR*) was introduced into the PDO300Δ*mucR* strain, and alginate production was restored and increased by about 4.4-fold compared with that of strain PDO300. The Δ*mucR* mutant harbouring only the vector pBBR1MCS-5 showed an about 1.6-fold increase in alginate production compared with strain PDO300 (Table 2).

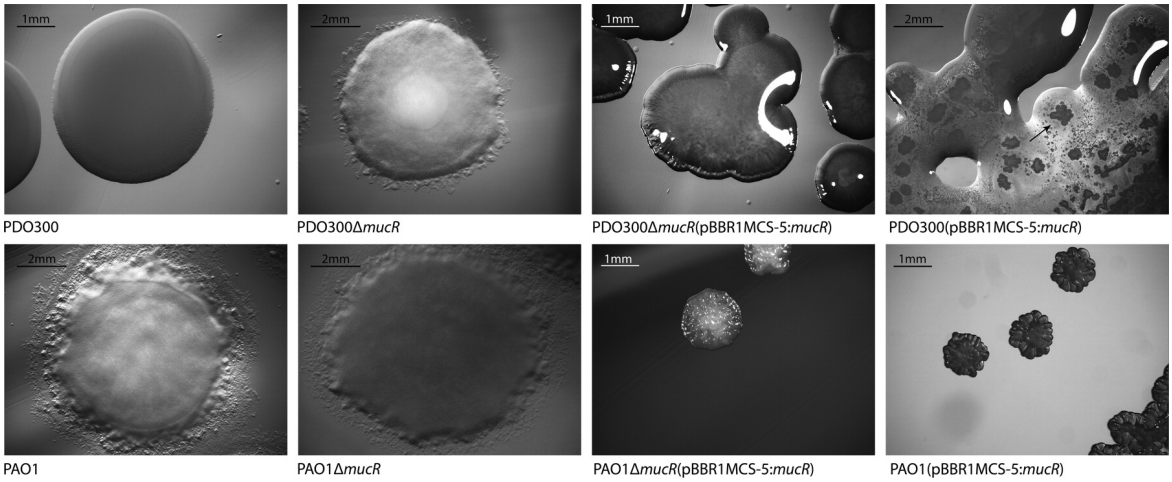

Figure 1. MucR is essential for the mucoid colony morphology. Colonies of various *P. aeruginosa* strains were grown on PIA medium for 24 h. The top row shows the mucoid strain PDO300 and the strains derived from it; the bottom row shows the nonmucoid strain PAO1 and the plasmids derived from it. Plasmids harbored by certain strains are indicated in parentheses.

Table 2: Alginate quantification of various *P. aeruginosa* strains

Strain	Alginate Production (g/g CDM[a])	SD[b]
PDO300	0.225	± 0.047
PDO300Δ*mucR*	0.006	± 0.001
PDO300 (pBBR1MCS-5)	0.584	± 0.023
PDO300Δ*mucR* (pBBR1MCS-5)	0.386	± 0.021
PDO300 (pBBR1MCS-5:*mucR*)	1.54	± 0.130
PDO300Δ*mucR* (pBBR1MCS-5:*mucR*)	0.958	± 0.170
PAO1	0	± 0
PAO1Δ*mucR*	0	± 0
PAO1 (pBBR1MCS-5)	0	± 0
PAO1Δ*mucR* (pBBRMCS-5)	0	± 0
PAO1 (pBBR1MCS-5:*mucR*)	0	± 0
PAO1Δ*mucR* (pBBRMCS-5:mucR)	0	± 0
PDO300 (pBBR1MCS-5:*wspR*)	0.295	± 0.006
PDO300 (pBBR1MCS-5:*rocR*)	0	± 0

[a] CDM, cellular dry mass
[b] SD, standard deviation. Results represent the data of four independent experiments

To assess whether the increase in alginate production was due to a general increase in intracellular c-di-GMP levels based on the presence of multiple *mucR* gene copies or was the result of a more specific function of MucR, additional c-di-GMP-influencing genes were introduced. The DGC gene (*wspR*/PA3702) shown to encode a highly active DGC was expressed in PDO300 (58, 88). This resulted not in an increase but in a twofold reduction in alginate production (Table 2). Overexpression of *rocR* (PA3947), which encodes a highly active PDE, abolished alginate production (88, 89) (Table 2).

MucR impacts colony morphology, auto-aggregation, pellicle formation, attachment to surfaces, and swarming motility. To further assess the function of MucR, the Δ*mucR* mutants were characterized with respect to several phenotypes known to be influenced by c-di-GMP levels. Strains harbouring additional copies of the *mucR* gene showed an altered phenotype. On solid medium these strains typically formed compact wrinkly colonies, which were surrounded by alginate when PDO300 was considered (Fig. 1). In liquid culture, cells harbouring plasmids encoding MucR formed large aggregates (pellicles) at the air-liquid interface (Fig. 2). In order to assess if any of these phenotypic changes were due to increased alginate production mediated by MucR, the Δ*alg8* mutant of PDO300, which is defective in alginate biosynthesis, was employed (137). Multiple copies of the *mucR* gene in the alginate-negative Δ*alg8* mutant resulted in small wrinkly colonies and the same auto-aggregation phenotype that was observed for the nonmucoid strain PAO1 (data not shown and Fig. 2).

The solid surface assay was applied to assess attachment and biofilm formation. In general, MucR mediated increases in initial attachment of about 8.4-fold and 8.9-fold when produced in PDO300 and PAO1, respectively (Table 3). These results were mimicked by continuous-culture flow cell biofilm analysis. Cellular layers, 1or 2 cells thick, were observed after 20 h on the glass surface for the PDO300 and PDO300Δ*mucR* strains (Fig. 3). Multiple copies of the *mucR* gene in PDO300 resulted in an initial increased attachment which developed into highly structured biofilms after 20 h (Fig. 3).

Recent studies have described a link between c-di-GMP levels and swarming motility in *P. aeruginosa* (87, 103). The alginate-overproducing strain PDO300 did not show a swarming phenotype, whereas PAO1 did. The lack of swarming shown in the PDO300 strains could be due to the presence of alginate. Thus, when the Δ*alg8* mutant of PDO300 was analysed, this mutant showed swarming motility (Fig. 4). Deletion of the *mucR* gene did not significantly change the level of swarming motility in any of the strains (Fig. 4). In strains harbouring only the vector and where gentamicin was added to the media, the swarming

phenotype was reduced but detectable (Fig. 4). The presence of plasmids containing the *mucR* gene in the PAO1, PAO1Δ*mucR*, and PDO300Δ*alg8* strains resulted in a strong inhibition of swarming motility (Fig. 4).

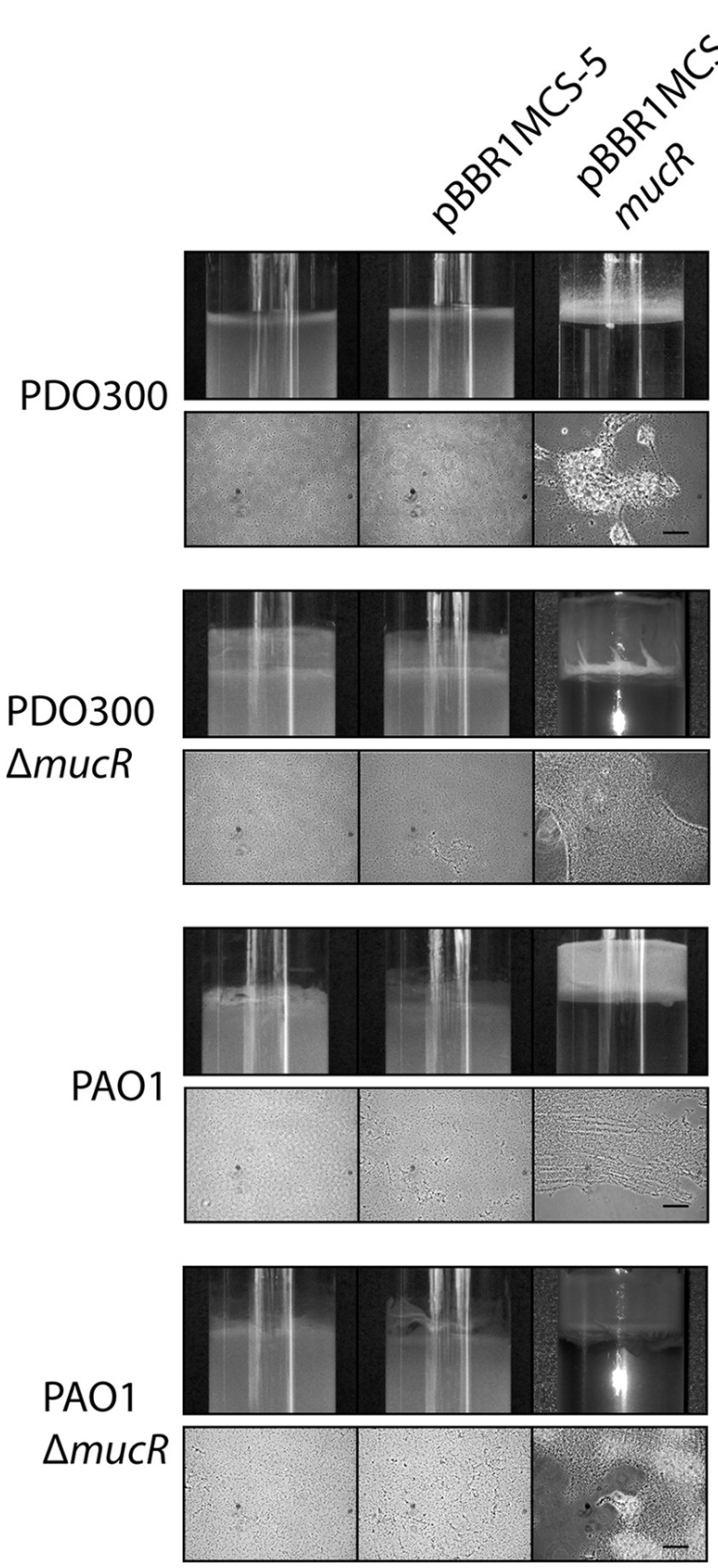

Figure 2. Multiple copies of *mucR* induce auto-aggregation and the formation of pellicles. Various strains were grown in LB for 16 h. The top row for each strain indicated on the left contains photographs of overnight cultures grown in LB overnight showing pellicle formation. The bottom row for each strain contains phase-contrast microscopic images showing the cell aggregation; the black bars represent 50 μm. Plasmids (where present) are indicated along the top.

Table 3: Rapid solid surface attachment assay

Strain	OD 550 nm	SD[a]
PDO300 (pBBR1MCS-5)	0.088	± 0.046
PDO300Δ*mucR* (pBBR1MCS-5)	0.198	± 0.179
PDO300Δ*mucR* (pBBR1MCS-5:*mucR*)	0.742	± 0.141
PAO1 (pBBR1MCS-5)	0.114	± 0.078
PAO1Δ*mucR* (pBBR1MCS-5)	0.095	± 0.028
PAO1Δ*mucR* (pBBR1MCS-5:*mucR*)	0.983	± 0.250

[a] SD, standard deviation. Results represent the data of three independent experiments

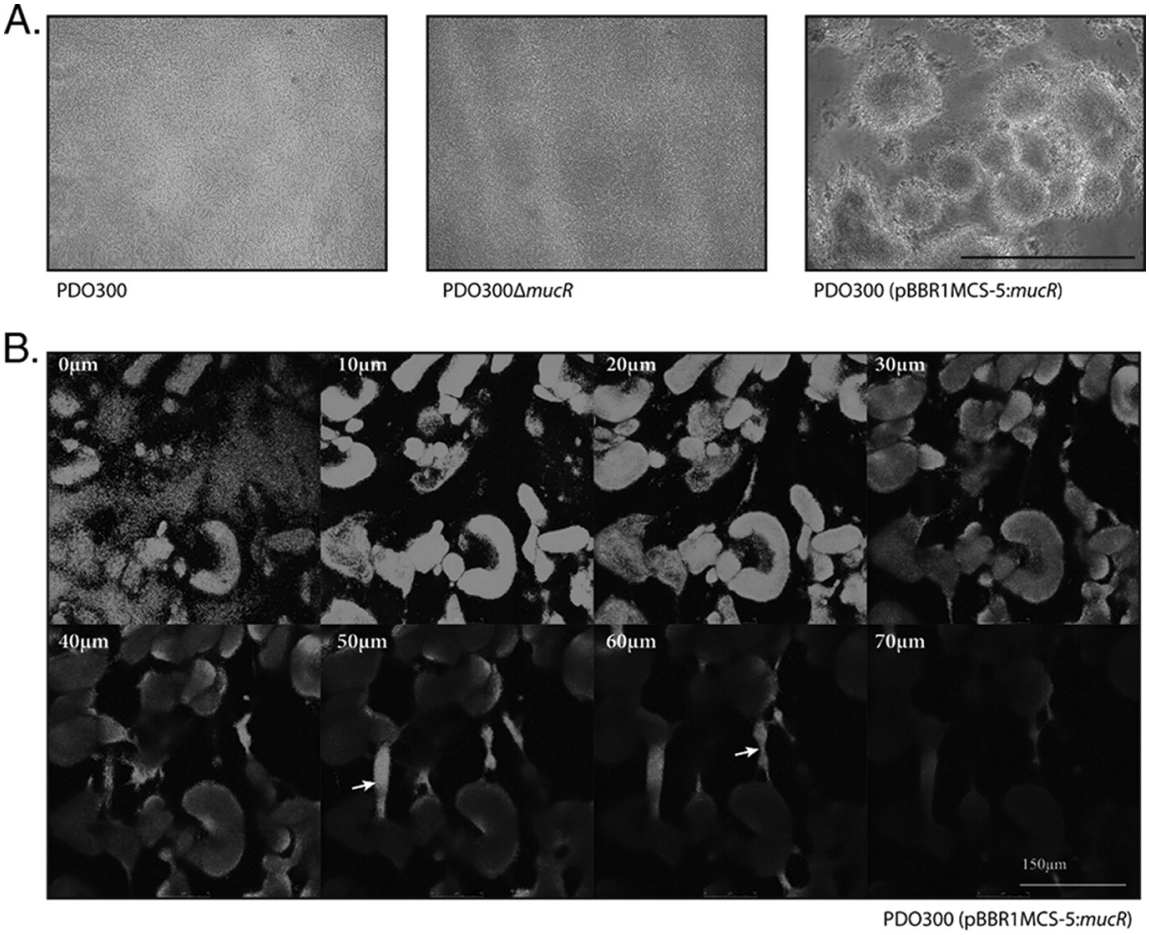

Figure 3. Multiple copies of *mucR* lead to the formation of highly structured biofilms. (A) Phase-contrast images of 24-h-old biofilms grown in a continuous-culture flow cell. Black bar represents 200 μm. (B) Confocal laser scanning microscopy images of a 24-h-old biofilm of the strain PDO300(pBBR1MCS-5:*mucR*). Each frame represents a picture 10 μm away from the last. The white bar represents 150 μm; the white arrow shows the formation of bridge-like structures connecting adjacent microcolonies.

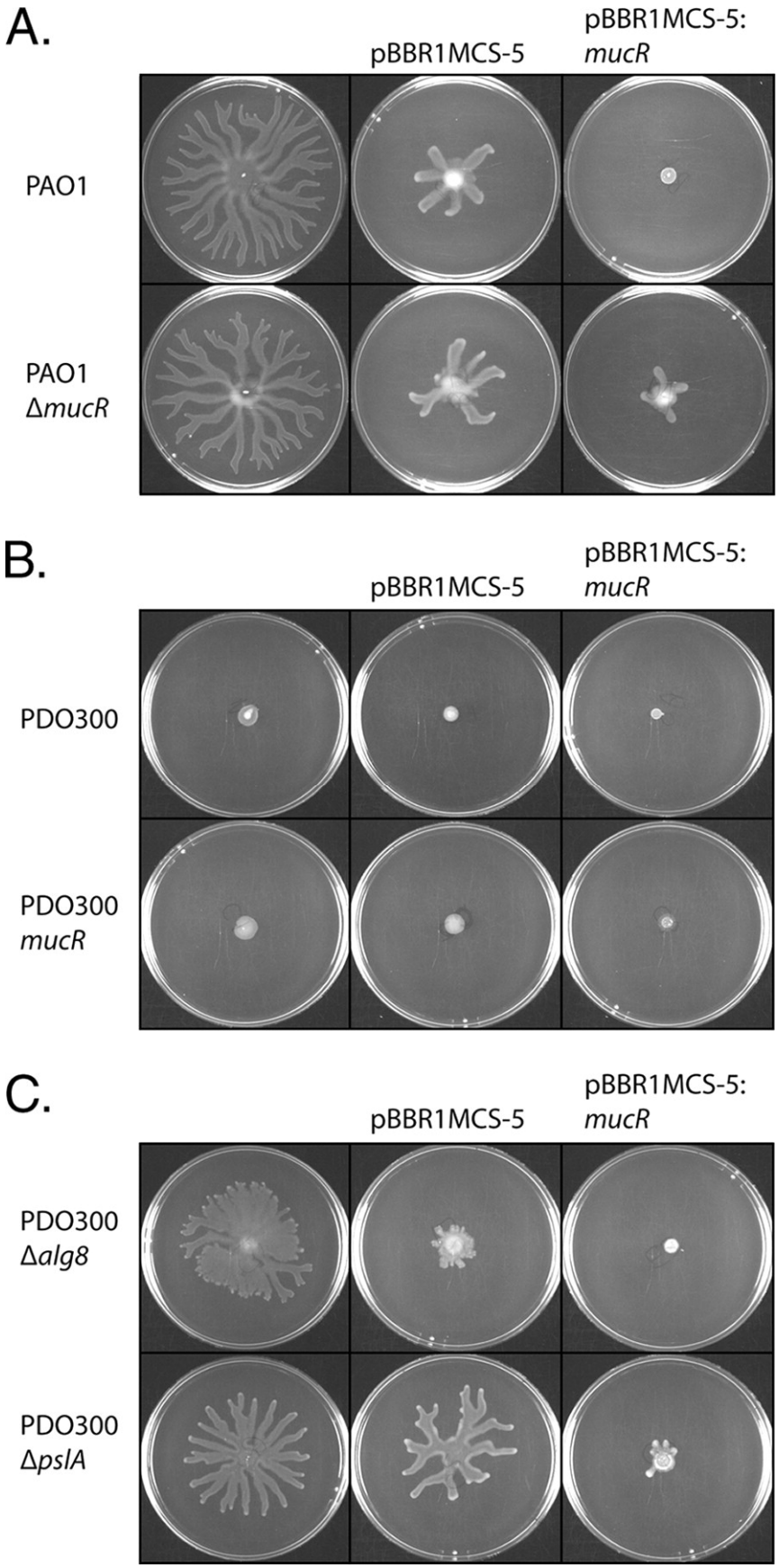

Figure 4. Loss of *mucR* does not influence swarming motility, but multiple copies of *mucR* inhibit swarming motility. (A) Swarming motility of the nonmucoid *P. aeruginosa* strain PAO1 and its isogenic *mucR* knockout mutant. (B) Swarming motility of the mucoid *P. aeruginosa* strain PDO300 and its isogenic *mucR* knockout mutant. (C) Swarming phenotype of a mutant derived from the mucoid PDO300 strain incapable of producing alginate due to the loss of the *alg8* gene, which is essential for alginate biosynthesis.

MucR affects the outer membrane protein profile. The altered phenotypes described above suggested that MucR influences cell surface properties. Thus, the outer membrane protein profiles were analysed. The protein profiles differed between the mucoid strain PDO300 and the nonmucoid strain PAO1 with respect to two proteins (Fig. 5). A protein with an apparent molecular mass of 56 kDa was present only in PDO300 and was identified as AlgE by using tryptic peptide fingerprinting employing MALDI-TOF mass spectrometry. AlgE is essential for alginate production, presumably enabling the export of alginate (128, 130). Another protein with an apparent molecular mass of 53 kDa was found in PAO1 and absent in PDO300 (Fig. 5). This protein was identified as FliC, a flagellin type B protein. In the Δ*mucR* mutant of PDO300, AlgE becomes less abundant while FliC becomes apparent. The PAO1Δ*mucR* showed no significant change in the outer membrane protein profile. Introduction of a plasmid encoding MucR significantly reduced the levels of FliC in the outer membrane of both PAO1 and PDO300 but did not affect the levels of AlgE in PDO300 (Fig. 5).

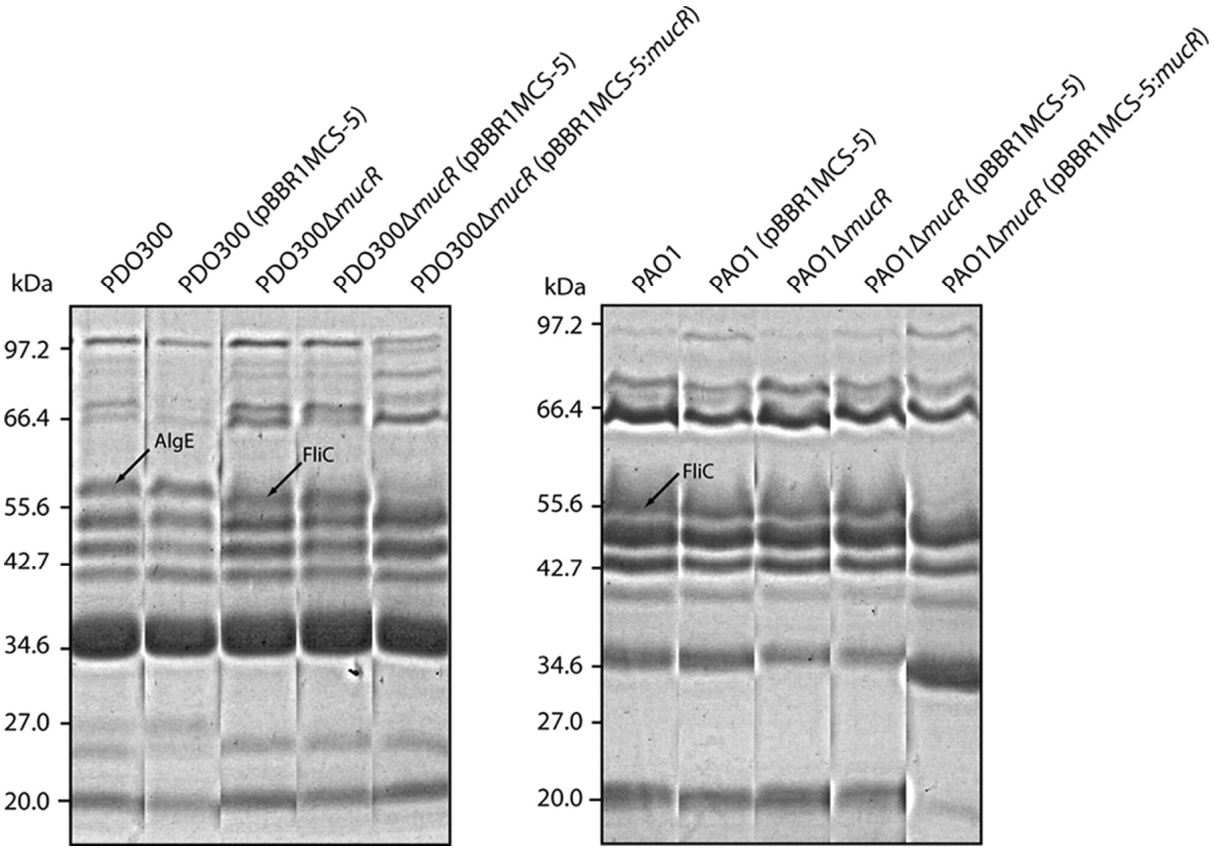

Figure 5. Outer membrane protein profiles of various *P. aeruginosa* strains. (Left) Outer membrane protein profiles of the Δ*mucR* mutant derived from PDO300; (right) outer membrane protein profiles of the Δ*mucR* mutant derived from PAO1. The arrows indicate the identity of proteins whose levels differed between mutants. Plasmids harbored by certain strains are indicated in parentheses.

The GGDEF/EAL domain is required for *mucR* function. To establish whether MucR function in the regulation of alginate production was dependent on the GGDEF/EAL domain containing the C terminus of MucR, a hybrid gene encoding only the first 277 amino acids of MucR was constructed. This region corresponded to the seven-transmembrane putative MHYT-sensing domain but lacked the GGDEF and EAL domains. This N-terminal region and full-length MucR were each translationally fused to the reporter enzymes LacZ and PhoA. Full-length MucR but not C-terminally truncated MucR restored alginate production to levels exceeding wild-type levels as well as mediated the formation of wrinkly colonies and an auto-aggregation phenotype when produced in the Δ*mucR* mutant (Fig. 6; data not shown).

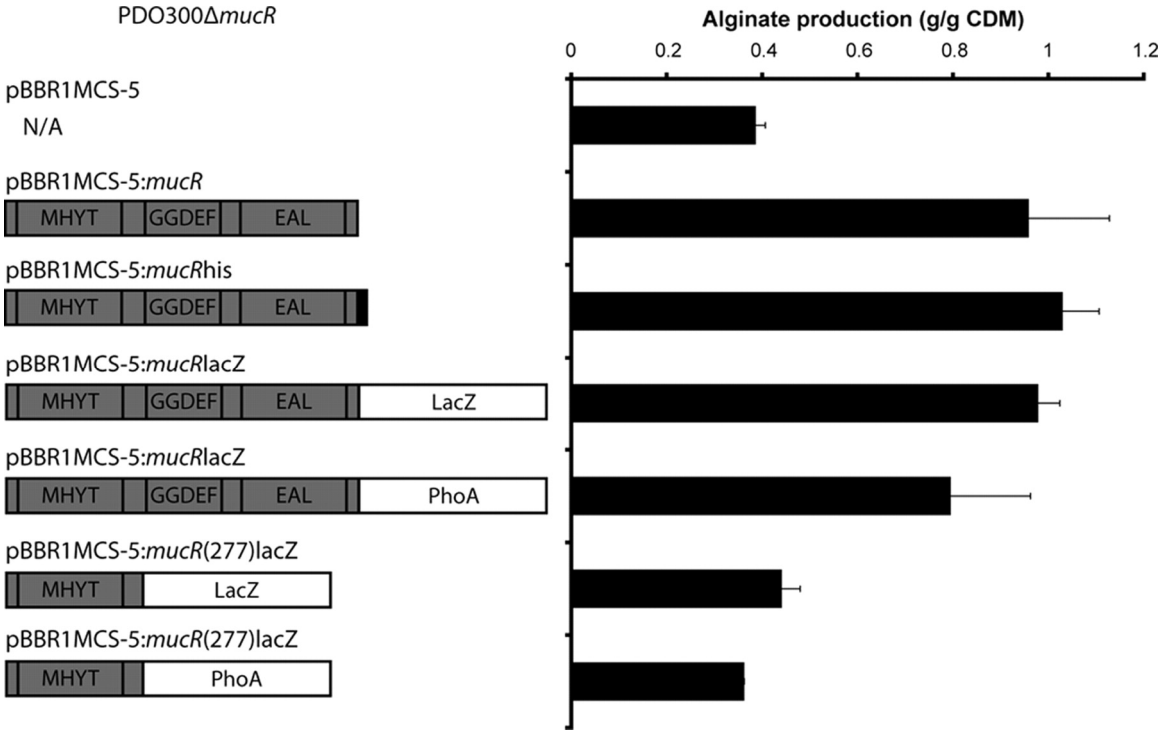

Figure 6. The alginate stimulating activity of MucR is dependent on the GGDEF and EAL domains containing the C terminus. (Left) Schematic representations of the various MucR fusion proteins. Plasmids harbored by the PDO300Δ*mucR* strain are indicated in parentheses. (Right) Alginate production levels of the PDO300Δ*mucR* strain harboring the genes encoding the various MucR fusion proteins. Right, CDM, cell dry mass. Alginate quantification data represent the results of three independent experiments.

Subcellular localization, membrane topology, and purification of MucR. The fusion proteins described above were employed to test the predicted membrane topology. *P. aeruginosa* cells expressing the various fusion proteins were fractionated into the cell lysate, soluble cytosol, and insoluble envelope fraction. No significant alkaline phosphatase activity could be detected for either the full-length or truncated MucR protein fused to PhoA in any of the fractions (Table 4). β-Galactosidase activity could be detected for both the full-length and truncated MucR-LacZ fusions, with the highest specific activity in the envelope fraction (Table 4).

Table 4. β-galactosidase and alkaline phosphotase activities of MucR fusion proteins in various subcellular fractions

	LacZ U(μM/min)/mg ± SD[b]	PhoA U(μM/min)/mg ± SD[b]
MucR lacZ / phoA WCL[a]	11.06 ± 0.77	0.01 ± 0.00
MucR lacZ / phoA SOL[a]	11.54 ± 1.42	0.01 ± 0.00
MucR lacZ / phoA MEM[a]	48.68 ± 0.22	0.03 ± 0.00
MucR(277) lacZ / phoA WCL[a]	135.45 ± 2.41	0.11 ± 0.01
MucR(277) lacZ / phoA SOL[a]	34.10 ± 3.90	0.12 ± 0.01
MucR(277) lacZ / phoA MEM[a]	559.40 ± 20.36	0.07 ± 0.00

[a] WCL, whole cell lysate; SOL, soluble; MEM, membrane.

[b] SD, Standard deviation. Results represent data of three independent experiments.

In addition, plasmid pBBR1MCS-5:*mucR*his was constructed, encoding C-terminally hexahistidine-tagged MucR. The PDO300Δ*mucR* strain harbouring pBBR1MCS-5:*mucR*his showed restored alginate production and was used to detect hexahistidine-tagged MucR via immunoblotting using anti-hexahistidine antibodies (Fig. 7). Immunoblot analysis of the cell lysate, cytosol, and envelope enabled detection of two protein bands in both the cell lysate and the envelope but not in the soluble cytosol fraction. These proteins showed an apparent molecular mass of 65 kDa and 50 kDa, respectively (Fig. 7). The theoretical molecular mass of full-length MucR is 74.4 kDa. The detected proteins could not be separated from the complex mixture of proteins in the gel. To confirm that these proteins corresponded to MucR, the solubilized total membrane fraction was subjected to Ni-NTA affinity purification, resulting in partial purification of the two detected proteins, enabling identification via tryptic peptide fingerprinting (Fig. 7). Both proteins were identified as MucR; however, only peptides corresponding to the C-terminal half of MucR and none from the transmembrane regions were detected. In addition, a protein with an apparent molecular mass of 330 kDa could be detected by immunoblotting, and peptide fingerprinting analysis confirmed that it belongs to MucR; this is presumably aggregate formation due to the hydrophobicity in the N terminus (Fig. 7).

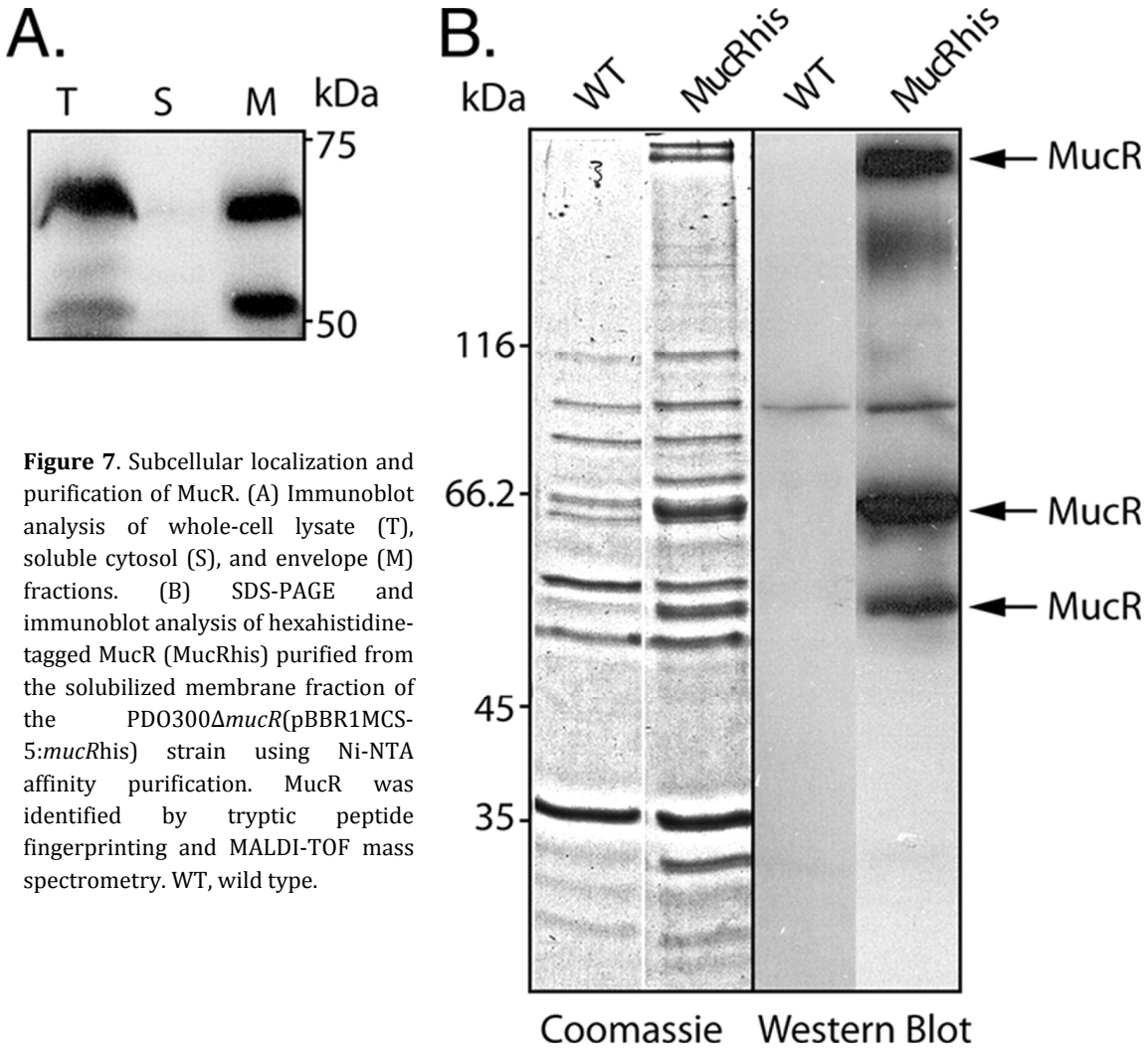

Figure 7. Subcellular localization and purification of MucR. (A) Immunoblot analysis of whole-cell lysate (T), soluble cytosol (S), and envelope (M) fractions. (B) SDS-PAGE and immunoblot analysis of hexahistidine-tagged MucR (MucRhis) purified from the solubilized membrane fraction of the PDO300Δ*mucR*(pBBR1MCS-5:*mucR*his) strain using Ni-NTA affinity purification. MucR was identified by tryptic peptide fingerprinting and MALDI-TOF mass spectrometry. WT, wild type.

Discussion

Recently it has been shown that c-di-GMP binding to membrane-anchored Alg44, an essential alginate biosynthesis protein, is required for alginate production in *P. aeruginosa* (3, 102, 135). It has been suggested that some of the DGC and PDE proteins respond to different signals by controlling intracellular c-di-GMP levels in localized pools. Thus, c-di-GMP sensing/binding proteins may be co-localized in the membrane with proteins producing and/or degrading c-di-GMP.

Experimental evidence to support this hypothesis had previously been obtained for *Gluconacetobacter xylinus*, where the DGC DgcA and the PDE PdeA were shown to co-purify with the c-di-GMP binding cellulose synthase (141), and for *Caulobacter crescentus*, where the GGDEF-containing protein PleD localized to the base of the flagellum where its DGC activity is required for flagellum ejection (117).

Twenty-two of the 38 DGCs and PDEs contain predicted transmembrane regions, and 14 of these contained domains recognized by Pfam (43). These domains were the PAS and PAC domains, involved in numerous signalling processes sensing diverse signals; the CHASE domains, sensing domains predicted to bind diverse low-molecular-weight ligands, such as the cytokinin-like adenine derivatives or peptides (109); the 7TM-DISM2 domains, thought to act as a receptor for carbohydrates (4); the MASE1 domain, with unknown function; and the MHYT domains, thought to sense oxygen, CO, or NO through the coordinated binding of one or two copper atoms (51). Both oxygen and NO have previously been shown to influence alginate production in *P. aeruginosa* (21, 46, 183, 184). Therefore, the two MHYT domain-containing proteins (MucR and PA3311) were considered. However, in a recent study, PA3311 showed no detectable DGC or PDE activity, whereas MucR showed DGC activity (88). Consequently, in this study MucR was functionally characterized.

The isogenic Δ*mucR* mutant of alginate-overproducing *P. aeruginosa* PDO300 showed a nonmucoid colony morphology and was strongly impaired in alginate production, and restoration of alginate production by the *mucR* gene suggested that MucR is involved in regulation of alginate biosynthesis (Fig. 1 and 2). The observed induction of alginate production by only the vector pBBR1MCS-5 had previously been described to be mediated by gentamicin (135, 137).

Introduction of the *mucR* gene-containing plasmid into PDO300 mediated a wrinkly colony morphology and strongly increased alginate production (Fig. 1 and 2). The small wrinkly colony morphology is very similar to the "small-colony variant" observed with the overexpression of the DGC WspR protein and commonly found among isolates from CF patients (34, 64).

The finding that the introduction of the *wspR* gene, encoding a cytosolic DGC showing 18.5-fold higher activity than MucR, did not cause an increased alginate production ((88); Table 2) supports the premise of a specific role of MucR in the regulation of alginate biosynthesis and the hypothesis of localized pools of c-di-GMP.

Previous studies showed that various c-di-GMP concentration-controlling enzymes, e.g., SadC and BifA, inversely regulate attachment/biofilm formation and swarming motility in *P. aeruginosa* (28, 80, 87, 103). In general, DGCs increase intracellular c-di-GMP levels and correlate with enhanced aggregation, attachment, and biofilm formation, but with repressed swarming motility, whereas PDEs lower levels of c-di-GMP and correlate with the contrary. Overproduction of MucR followed this trend, but loss of the *mucR* gene did not result in any change in these phenotypes, as would be expected of a DGC directly involved in regulation of these phenotypic properties.

Although transfer of pBBR1MCS-5:*mucR*, i.e., multiple *mucR* gene copies, into various *P. aeruginosa* strains led to multiple phenotypic changes, many of these phenotypic changes resemble the overexpression of many DGCs and the corresponding increase in c-di-GMP levels (34, 64, 103, 139, 140, 166).

Since MucR impacted phenotypic properties which often depend on proteinaceous cell surface structures, the outer membrane protein profiles of the various strains were analysed (Fig. 5). The strong decrease of the copy numbers of the flagella filament protein FliC in strains PAO1 and PDO300 harbouring plasmid pBBR1MCS-5:*mucR* correlated with the loss of swarming motility (Fig. 4). FliC has been described to be required for flagellum-based swarming motility (110). However, plasmid pBBR1MCS-5:*mucR* caused not an increase but a slight decrease of the copy numbers of the alginate export protein AlgE, while mediating alginate overproduction (Fig. 5).

The loss of MucR function after removal of the C-terminal region comprising the GGDEF domain suggested that DGC activity is required for MucR function (Fig. 6). As LacZ can fold correctly and become active only when exposed to the cytosol and as PhoA can correctly

fold as an active enzyme only in the periplasm (91), the reporter enzyme activities support the prediction that MucR is localized to the cytoplasmic membrane with its GGDEF/EAL C terminus exposed to the cytosol (Table 3).

Overall this study suggested that MucR is a membrane-anchored DGC which is a positive regulator of alginate biosynthesis. In accordance with the recent findings by Merighi et al. (102) that the c-di-GMP-binding PilZ domain of the Alg44 membrane protein is essential for alginate biosynthesis, a model for c-di-GMP-dependent regulation of alginate biosynthesis was developed (Fig. 8). The presence of the proposed localized c-di-GMP pool was supported by the finding that a single gene copy of *mucR* is required for normal levels of alginate biosynthesis in mucoid *P. aeruginosa*, whereas multiple copies of *mucR* led to phenotypes common to overexpression of DGCs (87). Further support that MucR regulated alginate biosynthesis via localized c-di-GMP pools in the vicinity of Alg44 was obtained by the finding that MucR but not the highly active cytosolic DGC WspR protein significantly increased alginate production (Fig. 8). The model proposed that an as yet unidentified signal (possibly CO, NO, or O2 (51)) is detected via the MHYT domain of MucR. Reception of this signal might influence the activity of MucR by modulating the activity of the DGC, and possibly the PDE, domain of MucR.

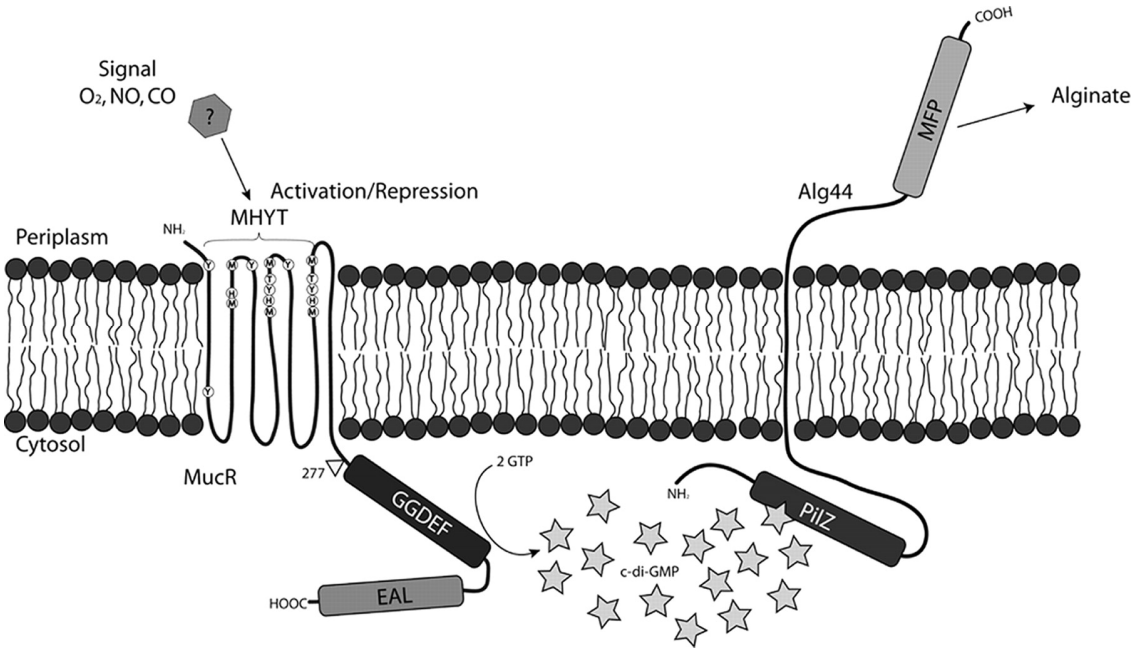

Figure 8. Proposed model for the MucR-mediated regulation of alginate biosynthesis in *P. aeruginosa*. The membrane topology and domain structure of MucR, as predicted by SMART and Pfam, are shown. The circles represent the conserved MHYT residues suggested to be involved in binding a copper atom and sensing oxygen, NO, or CO. The hexagon represents the putative signal. The stars represent c-di-GMP. Various domains are shown. MFP, membrane fusion protein domain, similar to multidrug efflux systems.

Acknowledgements

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Chapter V

Identification of a periplasmic AlgK-AlgX-MucD multiprotein complex in *Pseudomonas aeruginosa* involved in alginate biosynthesis and regulation

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Abstract

The opportunistic human pathogen *Pseudomonas aeruginosa* produces an extracellular polysaccharide called alginate. This is especially relevant in pulmonary infection of cystic fibrosis patients where it protects the bacteria from the hosts' immune system and the diffusion of antibiotics. Here a connection between the stability of a proposed alginate polymerisation/secretion complex and the regulation of the operon encoding these proteins was assessed. Experimental evidence was provided for a periplasmic multi-protein complex composed of AlgX, AlgK, and the regulatory protein MucD. Disruption of the alginate machinery in a mucoid strain, either by removal, or over production of various essential proteins resulted in an at least 2-fold increase in transcription of a *lacZ* reporter under the control of the *algD* promoter. Instability of the complex was indicated by an increase in secretion of alginate degradation products. This increase in transcription was found to be dependent on the negative regulatory protein MucD. Surprisingly, over production of MucD leads to a 3.3-fold increase in transcription from the alginate promoter and a 1.7-fold increase in the levels of alginate produced, suggesting an additional positive regulatory role for MucD in mucoid strains. Overall, this study provided experimental evidence for the proposed periplasmic multi-protein complex and established a link of a constituent of this complex, MucD, to transcriptional regulation of alginate biosynthesis genes.

Introduction

Pseudomonas aeruginosa is a clinically important opportunistic human pathogen, and its ability to produce a thick extracellular matrix predominantly composed of alginate significantly contributes to its pathogenicity. This is of particular relevance in cystic fibrosis (CF) patients where *P. aeruginosa* pulmonary infections are the leading cause of both morbidity and mortality (68).

The CF lung provides a unique environment to the pathogen which induces the overproduction of alginate by the bacteria, contributing to the clogging of the lung, while protecting the bacteria from the host immune response and antibiotic treatment (119, 159-161, 163). This switch to a mucoid phenotype is widely recognised as a poor prognosis indicator for patients, after which eradication of the infection is extremely unlikely. The exact mechanisms responsible for this switch are unclear but appear to involve a complex arrangement of transcriptional regulation, post-translational regulation and the mutation of hyper-mutable regions of the genome (127).

The 12 genes encoding the core alginate biosynthesis machinery are located in a single operon. AlgD and AlgA are involved in precursor synthesis; AlgI, AlgJ and AlgF are involved in acetylation of alginate (50); AlgG for epimerization (47); AlgL for cleavage (regulating the length of the polymer or degrading misguided alginate) (149); AlgE is an outer membrane export porin (66, 128); Alg8 is involved in polymerisation (134, 137); and Alg44 is involved in post-translational regulation (67, 135). The remaining two proteins, AlgK and AlgX, have unclear functions. These proteins are essential for the production of intact alginate (loss of AlgK or AlgX results in the secretion of short alginate degradation products) and are thought to play some sort of structural or protective role, guiding the alginate polymer through the periplasmic space (57, 74, 138). Recently, it has been suggested that AlgK may interact with the outer membrane protein AlgE (81). Interestingly, the purification of AlgX from *P. aeruginosa* resulted in the co-purification of the regulatory protein MucD which demonstrated the first interaction of proteins involved in alginate biosynthesis (57). MucD is a periplasmic protease involved in the regulation of alginate biosynthesis. It has been suggested that proteins predicted to be involved in polymerization–export of alginate form a complex spanning from the inner to the outer membrane with a bridging periplasmic protein scaffold (Fig. 1) (127).

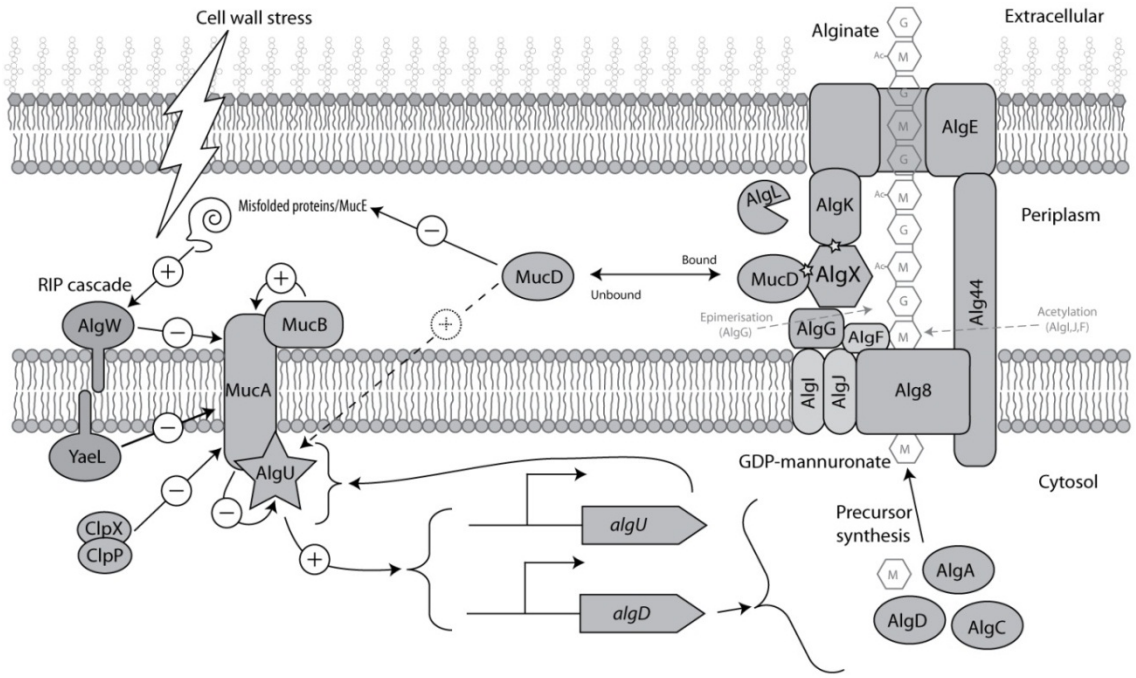

Figure 1: Schematic representation of predicted alginate biosynthesis complex and regulatory network. All interactions in the presented biosynthesis machinery are hypothetical except AlgX and MucD and AlgK and AlgX as indicated by *stars*. *Plus* and *minus symbols* indicate the effect the respective protein has on the subsequent protein in the pathway (not its overall regulatory role).

The alginate operon is under the tight control of a promoter upstream of *algD*, and transcription is initiated from this promoter via an alternate sigma factor AlgU (σ^{22}, AlgT). The *algU* gene is itself located in a partially auto-regulated operon called the "switch" loci containing the genes *algU*, *mucA*, *mucB*, *mucC* and *mucD* (25, 37, 45, 124). This region is a common site of mutations in clinical (mucoid) isolates, one study found that 80% of all clinical isolates contained mutations in this region (17). MucA, an intramembrane anti-sigma factor which sequesters AlgU at the membrane, is at the apex of a regulated intramembrane proteolysis (RIP) cascade (100, 152, 182). Several steps of this proteolytic cascade had been elucidated: MucB binds to the periplasmic side of MucA protecting it from proteolysis (97, 100). AlgW (*Escherichia coli* DegS homologue) is a periplasmic protease which is activated by the C-terminus of particular misfolded proteins (123) and cleaves the C-terminus of MucA which is subsequently cleaved on the cytosolic side by the intramembrane protease YaeL (MucP, PA3649) leading to the release of AlgU (23, 182). MucD, a homologue to the *E. coli* periplasmic serine protease DegP/HtrA, appears to be playing a role antagonistic to that of AlgW degrading misfolded proteins which would otherwise activate AlgW (14, 123, 181, 182). MucD also appears to be involved in response to stresses such as excessive heat or response to reactive oxygen species as MucD mutants showed increased sensitivity to H_2O_2 and heat killing (14).

Here evidence for the existence of a periplasmic multi-protein complex was provided. Furthermore, based on the apparent interaction between the regulatory protein, MucD, and other proteins of the alginate biosynthesis machinery, the relationship between the stability of the proposed alginate biosynthesis multi-protein complex and the transcriptional regulation of the alginate biosynthesis operon was investigated. Instability of the complex was achieved by removing or over producing proposed members of the complex. The activation of the alginate promoter in the presence and absence of MucD was assessed.

Materials and Methods

Bacterial strains and growth conditions. The bacterial strains, plasmids and oligonucleotides used in the present study are listed in Supplementary Table 1. *E. coli* strains were grown in LB medium at 37°C. *E. coli* strains S17-1 or SM10 was used for conjugative transfer of the suicide plasmids derived from pEX100T, the flipase encoding plasmid pFLP2 and the *φ*CTX-based integration vector mini-CTX-Palg-lacZ. Where required, antibiotics were used at the following concentrations: ampicillin 100 µg/ml, gentamicin 10 µg/ml and streptomycin 30 µg/ml. *P. aeruginosa* strains were grown in LB or PI(A) medium (Pseudomonas isolation [agar] medium—20 g of peptone, 10 g of K_2SO_4, 1.4 g $MgCl_2$, 0.025 g of triclosan and 20 ml of glycerol per litre) at 37°C. Where required, antibiotic concentrations used for *P. aeruginosa* strains were as follows: gentamicin 300 µg/ml carbenicillin 300 µg/ml and tetracycline 200 µg/ml. All chemicals were purchased from Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Isolation, analysis and manipulation of DNA. General cloning procedures were performed as described previously (146). pBBR1MCS-5, pTZ110 and pHERD-derived plasmids were transferred to *P. aeruginosa* strains via electroporation as previously described (27). DNA primers, dNTPs, Taq and Platinum Pfx polymerases were purchased from Sigma-Aldrich. DNA sequences of plasmid constructs were confirmed by DNA sequencing.

Construction and confirmation of deletion mutants. The *P. aeruginosa* deletion mutants for genes *alg8*, *alg44*, *algX* and *algE* were performed using the suicide plasmids described previously (57, 66, 135, 137). Suicide vectors for the construction of deletion mutants in the genes *mucD*, *algU* and *rpoN* were constructed as follows. Two regions upstream and downstream of the target gene were amplified using *Pfx* polymerase resulting in the fragments mucDN (comprising bases −2 to 406 relative to the designated mucD coding region followed by a *Bam*HI site) and mucDC (bases 1,008 to 1,410 preceded by a *Bam*HI), algUN (bases −164 to 260 relative to the first start codon flanked by a *Sca*I site and *Bam*HI site) and algUC (bases 797 to 1,217 flanked by a *Bam*HI site and *Sca*I site) and rpoNN (bases −15 to 389 flanked by a *Eco*RV site and *Bam*HI site) and rpoNC (bases 1,118 to 1,503 flanked by a *Bam*HI site and *Eco*RV site). Both PCR products were hydrolyzed by using *Bam*HI and inserted into the vector pGEM-T Easy (Promega, Madison, WI, USA). Vector pPS856 (69) was hydrolyzed with *Bam*HI and the fragment containing the aacC1 gene (encoding gentamicin acetyltransferase) flanked by two FRT (Flp

recombinase target) sites was inserted into the *Bam*HI site between the two regions of the target gene. The fragments comprising the gentamicin cassette flanked by the upstream and downstream regions of the genes of interest were then released by hydrolysis with *Sma*I (for *mucD*), *Eco*RI (for *rpo*N) or *Sca*I (for *alg*U) and inserted into *Sma*I site of vector pEX100T (69), resulting in plasmids pEX100T:ΔmucDΩGm, pEX100T:ΔalgUΩGm and pEX100T:ΔrpoNΩGm.

These suicide plasmids were transferred into *P. aeruginosa* and transconjugants were selected on mineral salt medium (150) containing gentamicin and 5% (wt/vol) sucrose. Cells growing on this selective medium should have emerged from double crossover events. Gene replacement was confirmed after subculture of cells on PIA medium containing gentamicin and using PCR with primers upstream and downstream of the homologous regions.

The Flp recombinase encoding vector pFLP2 (69) was transferred into *P. aeruginosa* ΩGm strains and grown on PIA containing carbenicillin for 12 h. The pFLP2 vector was later cured from the strain by growth on PIA medium containing 5% (wt/vol) sucrose. Gentamicin- and carbenicillin-sensitive cells were analysed by PCR for loss of the gentamicin-resistant cassette. Strains with mutations in multiple genes were constructed in a stepwise manner, deleting the genes sequentially in the order they are named in the strain.

To confirm that construction of the deletion mutants did not result in any polar effects, deletion mutants were complemented in *trans* with a plasmid containing the open reading frame of the deleted gene. Plasmids for the complementation of the *alg8*, *alg44*, *algX* and *algE* deletion mutants (pBBR1MCS-5:*alg8*, pBBR1MCS-5:*alg44*, pBBR1MCS-5:*algX* and pBBR1MCS-5:*algE*) are described in previous studies (57, 66, 135, 137). Plasmids for the complementation of the *mucD*, *algU* and *rpoN* deletion mutants were constructed as follows: the *mucD*, *algU* and *rpoN* open reading frames were amplified using the primers mucD-F-HiSDNd and mucD-R-*Sac*I; algU-F-HiSDNd and algU-R-*Bam*HI; and rpoN-F-HiSDNd and rpoN-R-*Bam*HI, respectively. The fragments were hydrolysed with *Hin*dIII and *Sac*I (*mucD*) and *Hin*dIII and *Bam*HI (*algU* and *rpoN*) and ligated into corresponding sites in the broad host range plasmid pBBR1MCS-5 (85), resulting in the plasmids pBBR1MCS-5:*mucD*, pBBR1MCS-5:*algU* and pBBR1MCS-5:*rpoN*.

Activation of the alginate promoter. Activation of the alginate promoter in deletion mutants was assessed using the plasmid pTZ110:Palg, and this plasmid contains

lacZ under the control of the *algD* promoter. The promoter region, located at −854 to 1 bp relative to the *algD* open reading frame, was amplified using the primers Palg-F-HiNo and Palg-R-Ba. The product was hydrolysed with *Hin*dIII and *Bam*HI and ligated into the *Hin*dIII and *Bam*HI sites of the plasmid pTZ110 (153). This plasmid was then transferred to the *P. aeruginosa* strain of interest.

Additionally, to assess the effect of artificially increasing the copy number of various genes (in *trans*) involved in alginate biosynthesis, strains were constructed with the same transcriptional reporter (*lacZ*) fusion described above integrated into the genome. These strains were created using an integration proficient *φ*CTX-based plasmid. The promoter region was amplified as described above and ligated into the *Hin*dIII and *Bam*HI sites of the plasmid mini-CTX-*lacZ* (9), resulting in the plasmid mini-CTX:Palg-lacZ. This plasmid was then transferred to the *P. aeruginosa* strain of interest and subsequently plated on PIA media containing tetracycline to select for colonies that have undergone recombination with the plasmid and subsequent integration of the alginate promoter reporter along with the tetracycline resistance gene. Integration at the *attB* site was confirmed by PCR using the primers Pser-down and Pser-up.

Activity of these transcriptional reporter promoter fusions was assessed as follows: strains were grown on PI(A) agar plates containing 200 µg/ml carbenicillin for 48 h. A representative sample of the bacterial lawn was scraped from the agar surface, washed three times in TBS (pH 7.8) and resuspended to an OD_{600} of approximately 0.2–0.3. The β-galactosidase activity of this was measured using a modified Miller method (105, 190). Briefly, 20 µl of the cells was added to 80 µl of permeabilisation solution (0.8 mg/ml hexadecyltrimethylammonium bromide, 0.4 mg/ml sodium deoxycholate, 5.4 µl/ml β-mercaptoethanol, 100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$) and incubated at 30°C for 15 min. Six hundred microlitres of the substrate solution (1 mg/ml *o*-nitrophenyl-β-d-galactoside, 2.7 µl/ml β-mercaptoethanol, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4). After a yellow colour has developed, the reactions are stopped with the addition of 700 µl stop solution (1 M Na_2CO_3) and time recorded. The OD_{420} was recorded and Miller units calculated (105).

Construction of a conditional non-mucoid PDO300 strain. PDO300 is an isogenic derivative of PAO1 with a non-functional *mucA* gene (muc22A allele). As the deletion mutants of *mucD* in the PDO300 and PDO300Δ*algX* background were seemingly not possible, a strain with the WT *mucA* gene under the control of the *araBAD* promoter (P_{BAD}) was constructed. The mucA gene was amplified using the primers mucA-F-SDNd

and mucA-R and ligated into the pGEM-T Easy cloning vector (Promega, WI, USA). The resulting plasmid was hydrolysed with *Nco*I and *Sal*I and ligated into the corresponding sites on the arabinose-inducible vector pHERD26T (121), resulting in pHERD26T:mucA. A 4,274-bp fragment containing the *araC* gene and *mucA* under the control of the P_{BAD} promoter was hydrolysed from the plasmid pHERD26T:mucA with *Nhe*I, and the resulting 5′ overhangs were filled in with T4 DNA polymerase (blunted). This fragment was hydrolysed with *Sal*I and the 1,576-bp araC-P_{BAD}-MCS containing fragment was ligated into a *Sal*I and *Sma*I hydrolysed integration proficient vector mini-CTX2 (70), resulting in mini-CTX2P_{BAD}:mucA.

mini-CTX2P_{BAD}:mucA was transferred by conjugation to the strains PDO300 and PDO300Δ*algX*. Transconjugates were selected for on tetracycline containing media and integration confirmed as described above, resulting in the strains PDO300-CTX2P_{BAD}:mucA and PDO300ΔalgX-CTX2P_{BAD}:mucA.

Alginate production assays. Alginate was harvested and purified as described previously (67). Uronic acid content was assessed through a modification of the Blumenkrantz and Asboe-Hansen (12) method described previously using 100% alginic acid from brown algae (Sigma-Aldrich) as a standard.

Free/dialysable uronic acids (alginate degradation products) were measured in the supernatant of 2 ml of overnight cultures. Briefly, total uronic acid content of the supernatant was determined, the supernatants were filtered with Amicon Ultra-0.5 (Millipore) centrifugal filters (nominal molecular weight cut-off 10 kDa) and the flow through was collected (containing free uronic acids and short length alginate degradation products) and the uronic acid content determined.

Purification of MucD/AlgX and pull-down experiments. A C-terminal translational fusion or MucD to a hexahistadine tag was constructed: *mucD* was amplified with the primers mucD-F-HiSDNd and mucD-R-6×His-*Bam*HI. This was hydrolysed with *Hin*dIII and *Bam*HI and ligated into corresponding sites in the plasmid pBBR1MCS-5, resulting in pBBR1MCS-5:mucDHis. This plasmid was introduced into the *P. aeruginosa* strain PAO1ΔmucD and PAO1ΔmucDΔalgX and the *E. coli* strain Rosetta 2 (Novagen). The *P. aeruginosa* strains were grown in 500 ml of rich media (32 g l^{-1} tryptone, 20 g l^{-1} yeast extract, 5 g l^{-1} NaCl) at 37°C for 24 h. Rosetta 2 strains were grown in 500 ml of LB at 37°C until an OD_{600} of 0.6 was reached, after which expression was induced with the addition of IPTG to a final concentration of 1 mM. Cells were harvested and washed three times in one

volume of TBS (pH 7.8) and suspended in 1/10th volume of lysis buffer (150 mM NaCl, 100 mM Tris–HCl, 0.2% Triton X-100, pH 8.0) with 1 mg ml^{-1} lysozyme and 1 mg ml^{-1} DNase. This was incubated for 20 min at 4°C with shaking and subsequently lysed by sonication. Insoluble cell debris was removed by centrifugation at 16,000×*g* for 20 min at 4°C. His-tagged MucD was purified from the lysate using TALON™ DynaBeads® (Invitrogen).

C terminally strep-tagged AlgX was purified as follows: The plasmid pBBR1MCS-5:algXStrep (57) was introduced into *P. aeruginosa* strains PDO300ΔalgX, PAO1ΔalgX, PAO1ΔmucDΔalgX and *E. coli* strain Rosetta 2. Cell lysates were prepared as described above. Supernatants were subjected to affinity purification with a Strep-Tactin® Superflow™ 1 ml Column (Novagen) according to the manufacturer's instructions.

Where the protease activity was to be assessed, purifications were completed in the absence of any protease inhibitors.

Analysis of proteins. Protein concentrations were determined using the Quant-iT™ Protein Assay Kit (Invitrogen). Proteins were separated by SDS-PAGE on 10% acrylamide gels. Bands of interest were identified by tryptic peptide fingerprinting using matrix-assisted laser desorption ionisation-time of flight/mass spectrometry (MALDI-TOF/MS) by the Centre for Protein Research at the University of Otago.

Analysis of protease activity. The protease activity of various protein extracts was assayed using Universal protease substrate (resorufin-labelled casein) (Roche) according to the manufacturer's instructions. Fifty micrograms of total protein from the purified MucDHis containing fractions was assayed with the addition of buffer or 150 µg of BSA, purified AlgX containing fractions or total membrane fractions from mucD deletion mutants. These were incubated for 120 min and the amount of free resorufin was then assessed by measuring the absorbance at 574 nm.

Gel filtration chromatography. One milligram of total protein in 500 µl was loaded on to a Superdex 200 10/300GL column. Two column volumes of lysis buffer (with 0.02% Triton X-100 instead of 2%) was passed through the collum at 0.5 ml min^{-1}. The absorbance at 280 nm was monitored. Fractions were collected in 0.5 ml steps and subsequently assessed by SDS-PAGE.

Results

Generation and characterisation of *algX/mucD* mutants. To better understand any interaction between MucD and AlgX, we attempted to generate a set of deletion mutants for these two genes in both non-mucoid (PAO1) and mucoid (PDO300) parent strains. Disruption of *mucD* in PAO1 led to a mucoid phenotype (PAO1Δ*mucD*) with alginate levels similar to that of the mucoid strain PDO300 (Table 1). The non-mucoid phenotype could be restored by providing the *mucD* gene in *trans*. This is consistent with previous findings (14, 181, 189). Disruption of *algX* in the non-mucoid strain did not visibly alter the phenotype of the parent. Disruption of both *algX* and *mucD* in the non-mucoid parent (PAO1ΔmucDΔalgX) resulted in a non-mucoid strain, though short, dialyzable, uronic acid-containing molecules, i.e. alginate degradation products, could be detected in the culture supernatant. As would be expected, providing *algX* in *trans* to the double mutant resulted in a mucoid phenotype whereas providing *mucD* alone or both *mucD* and *algX* resulted in a non-mucoid phenotype (Table 1). It should be noted that both PAO1ΔmucD and more dramatically PAO1ΔmucDΔalgX showed impaired growth characteristics, growing slower and reaching cell densities about 0.73 and 0.38 times less than that of wild type, respectively. These strains also appeared to be more susceptible to lysis during washing with TBS. This growth could be restored to wild-type levels when complemented with *mucD* or *mucD* and *algX* in *trans*. All other strains had similar growth rates (data not shown).

Disruption of *algX* in the mucoid parent (PDO300ΔalgX) resulted, as previously described (57, 138), in a non-mucoid phenotype with the secretion of free uronic acids. Alginate production could be restored by providing *algX* in *trans* (Table 1).

Table 1: Alginate production of Δ*algX* and Δ*mucD* deletion mutants

Strain	Alginate / CDW (g/g) ± SD	% dialyzable free uronic acids ± SD
PDO300 (MCS-5)	1.46 ± 0.35	16.45 ± 4.32
PDO300ΔalgX (MCS-5)	ND	100 ± 5.34
PAO1 (MCS-5)	ND	ND
PAO1ΔalgX (MCS-5)	ND	ND
PAO1ΔmucD (MCS-5)	1.59 ± 0.28	12.24 ± 5.28
PAO1ΔmucDΔalgX (MCS-5)	ND	100 ± 4.28
PDO300ΔalgX (MCS-5:algX)	1.67 ± 0.29	52.60 ± 7.89
PAO1ΔalgX (MCS-5:algX)	ND	ND
PAO1ΔmucD (MCS-5:mucD)	ND	ND
PAO1ΔmucDΔalgX (MCS-5:algX)	1.20 ± 0.17	25.01 ± 8.66
PAO1ΔmucDΔalgX (MCS-5:mucD)	ND	ND
PAO1ΔmucDΔalgX (MCS-5:algXmucD)	ND	ND

ND = not detected

Intriguingly, although multiple attempts were made to disrupt *mucD* in the mucoid strain PDO300 and its isogenic Δ*algX* strain, no mutants could be generated. PDO300 is an isogenic derivative of PAO1 generated through the replacement of *mucA* with the defective mucA22 allele (from the clinical isolate FRD1) containing a single base pair deletion in a string of guanine residues which results a premature stop codon and a truncated MucA missing 48 residues from its periplasmic C-terminus (99). In order to address this issue, we attempted to mimic the PAO1 environment by providing PDO300 with a conditionally expressed functional WT *mucA* and attempt to disrupt *mucD* in this strain. Accordingly, the full-length *mucA* (under the control of the arabinose-inducible pBAD promoter) was integrated into the genome via the CTX2 vector, which resulted in strains PDO300-CTX2P_{BAD}:mucA and PDO300ΔalgX-CTX2P_{BAD}:mucA. PDO300-CTX2P_{BAD}:mucA showed a mucoid colony morphology in the absence of arabinose and a non-mucoid colony morphology in the presence of 0.5% arabinose (data not shown). Attempts were made to knock out *mucD* in these strains in the presence of various concentrations of arabinose with no success. Further attempts to disrupt *mucD* in another mucoid strain, the clinical isolate FRD1, also proved unsuccessful.

Experimental evidence for periplasmic multi-protein complex composed of AlgK, AlgX and MucD. To provide experimental evidence for the long-time proposed periplasmic multi-protein complex and its implications to regulation of alginate

biosynthesis, further analysis of protein–protein interactions was conducted. Hexahistadine-tagged MucD was purified from strains PAO1ΔmucD(pBBR1MCS-5:mucD6xHis) and PAO1ΔmucDΔalgX(pBBR1MCS-5:mucD6xHis) using TALON Dynabeads (Fig. 2a). One primary band corresponding to full-length mature MucD and three truncations of MucD were observed and identified by MALDI-TOF/MS. Both of these extracts showed similar levels of protease activity as measured using resorufin-labelled casein. Strep II-tagged AlgX was enriched from PDO300ΔalgX(pBBR1MCS-5:algXstrep), PAO1ΔalgX(pBBR1MCS-5:algXstrep) and PAO1ΔmucDΔalgX(pBBR1MCS-5:algXstrep) cell lysates using Strep-Tactin superflow columns. Several co-eluting proteins could be detected and were identified by MALDI-TOF/MS (Fig. 2). In purified fractions from PDO300ΔalgX(pBBR1MCS-5:algXstrep), three bands were identified as the essential alginate biosynthesis protein AlgK; one at 49.5 kDa corresponding to the mature full-length protein; and two truncations of AlgK, one at 45.6 kDa and one at 43.7 kDa, and the previously described co-eluting protein MucD could be identified in two protein bands 47.8 kDa (full-length) and 43.7 kDa (truncated) (Fig. 2a). Several other biotin-containing proteins were present (data not shown). In purified fractions from PAO1ΔalgX(pBBR1MCS-5:algXstrep), only AlgX and MucD could be identified with AlgX being in much lower quantities than when purified from PDO300ΔalgX. From PAO1ΔmucDΔalgX(pBBR1MCS-5:algXstrep), only very small amounts of strep-tagged AlgX and full-length AlgK could be identified (Fig. 2a).

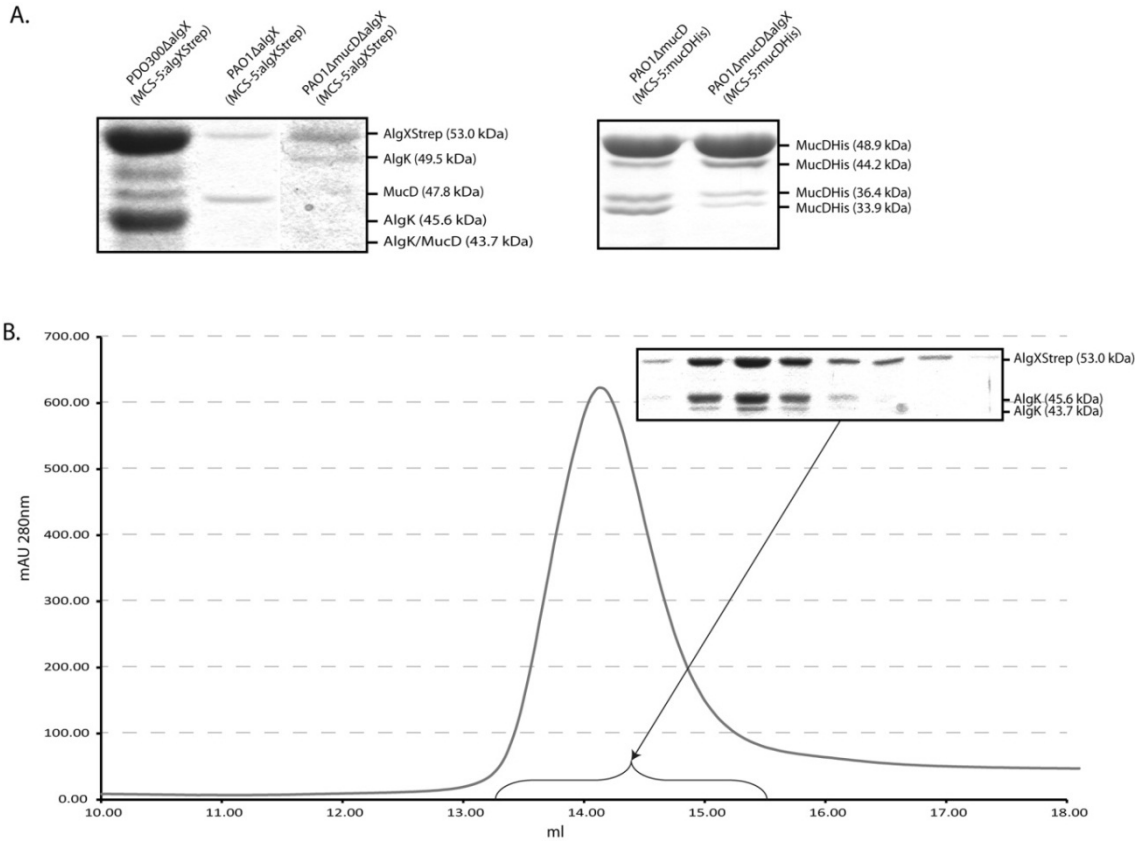

Figure 2: a Purification of strep-tagged AlgX and hexahistadine-tagged MucD from various *P. aeruginosa* strains. SDS-PAGE gel shows AlgX and co-eluting proteins. **b** Gel filtration chromatography (OD_{280nm}) of the strep-tagged AlgX purification from *P. aeruginosa* PDO300- ΔalgX(pBBR1MCS-5:algXStrep). *Inset* SDS-PAGE of peak of interest. All protein identities were confirmed by MALDI-TOF/MS

A purified AlgX fraction from PDO300ΔalgX(pBBR1MCS-5:algXstrep) was separated by gel filtration chromatography. One major peak could be detected at approximately 14 min (approximately 70 kDa) with a lower molecular weight shoulder. SDS-PAGE and subsequent MALDI-TOF/MS of the protein bands showed that this peak was composed of full-length strep-tagged AlgX and the two shorter truncations of AlgK. The shoulder was composed of strep-tagged AlgX alone (Fig. 2b).

As MucD is a serine protease, we assessed the influence of AlgX, the AlgX–AlgK complex or envelope fractions containing members of the alginate biosynthesis machinery had on the protease activity of MucD. MucD was purified from PAO1ΔmucDΔalgX(pBBR1MCS-5:mucDHis). Approximately 3 times molar excess of AlgX purified from *E. coli* Rosetta 2 (pBBR1MCS-5:algXStrep) or the AlgX–AlgK complex purified from PAO1ΔmucDΔalgX(pBBR1MCS-5:algXStrep) was added to the purified MucD. Neither AlgX nor the AlgX–AlgK complex had any significant effect on protease activity when compared

to BSA as control. This was repeated with crude envelope fractions from various strains, and again no effect on protease activity could be detected (Supplementary Table 2).

Loss of members of the alginate polymerase machinery affects the activation of the alginate promoter. Due to the previously described interaction between AlgX, a proposed member of the alginate synthesis/secretion machinery, and MucD, a regulatory protein involved in the transcriptional regulation of alginate biosynthesis (57), we assessed whether the loss of various members of the alginate biosynthesis machinery would have an effect on the levels of expression from the alginate promoter.

The plasmid pTZ110:Palg was constructed which contains the *lacZ* gene under the control of the promoter upstream of the alginate operon. This plasmid was introduced into various strains with disruptions in genes involved in the synthesis or secretion of alginate. The β-galactosidase activity and levels of alginate production were assessed. In the mucoid strain PDO300, disruption of *alg8* and *alg44* led to 2.5- and 4-fold reductions in alginate promoter activity, respectively, whereas disruption of *algX* or *algE* resulted in a 2.2-fold increase in alginate promoter activity (Table 2). As previously demonstrated, these mutants did not produce alginate, although dialyzable uronic acids could be detected in the Δ*algX* and Δ*algE* mutants.

Disruption of these same genes had no effect on the minimal levels of transcription and alginate production observed in the non-mucoid strain PAO1. Interestingly, although the mucoid strain PAO1ΔmucD produced alginate at levels equivalent to PDO300, the activity of the alginate promoter was about 6.5 times less in the PAO1ΔmucD strain. Disruption of both *mucD* and *algX* in PAO1 resulted in a 5-fold increase in promoter activity over PAO1ΔmucD (Table 2).

Table 2: Alginate promoter activity and alginate production of various alginate biosynthesis gene deletion mutants

Strain	Alginate promoter Miller units ± SD	Alginate / CDW (g/g) ± SD	% dialyzable free uronic acids ± SD
PDO300 (pTZ110:Palg)	17811.71 ± 1177.64	1.91 ± 0.22	16.45 ± 4.32
PDO300ΔalgX (pTZ110:Palg)	38040.60 ± 4752.08	ND	100 ± 5.34
PDO300Δalg8 (pTZ110:Palg)	6834.06 ± 970.73	ND	ND
PDO300Δalg44 (pTZ110:Palg)	4627.75 ± 274.74	ND	ND
PDO300ΔalgE (pTZ110:Palg)	39104.70 ± 3789.55	ND	100 ± 6.82
PAO1 (pTZ110:Palg)	65.96 ± 6.42	ND	ND
PAO1ΔalgX (pTZ110:Palg)	57.08 ± 17.08	ND	ND
PAO1ΔmucD (pTZ110:Palg)	2784.76 ± 584.85	1.54 ± 0.12	12.24 ± 5.25
PAO1ΔmucDΔalgX (pTZ110:Palg)	14349.76 ± 764.98	ND	100 ± 4.08

Artificially increasing the levels of alginate biosynthesis proteins leads to increased transcription from the alginate promoter when AlgX and MucD are present. To further assess the influence the alginate biosynthesis machinery has on the levels of transcription from the alginate promoter, various proteins essential for alginate biosynthesis were overproduced. To do this, the *lacZ* alginate promoter reporter fusion was integrated into the chromosome via the integration proficient mini-CTX2 vector. This allowed us to artificially increase the copy number of genes of interest by providing them on plasmids. It should be noted that the chromosomal promoter *lacZ* reporter reported significantly lower β-galactosidase activity than the plasmid born reporter (pTZ110:Palg), but the relative levels of transcription and alginate production were similar in the strains $PDO300_{CTXPalglacZ}$, $PDO300\Delta algX_{CTXPalglacZ}$, $PAO1CTXP_{alglacZ}$, $PAO1\Delta mucD_{CTXPalglacZ}$ and $PAO1\Delta mucD\Delta algX_{CTXPalglacZ}$ to those carrying the pTZ110:Palg (Fig. 3).

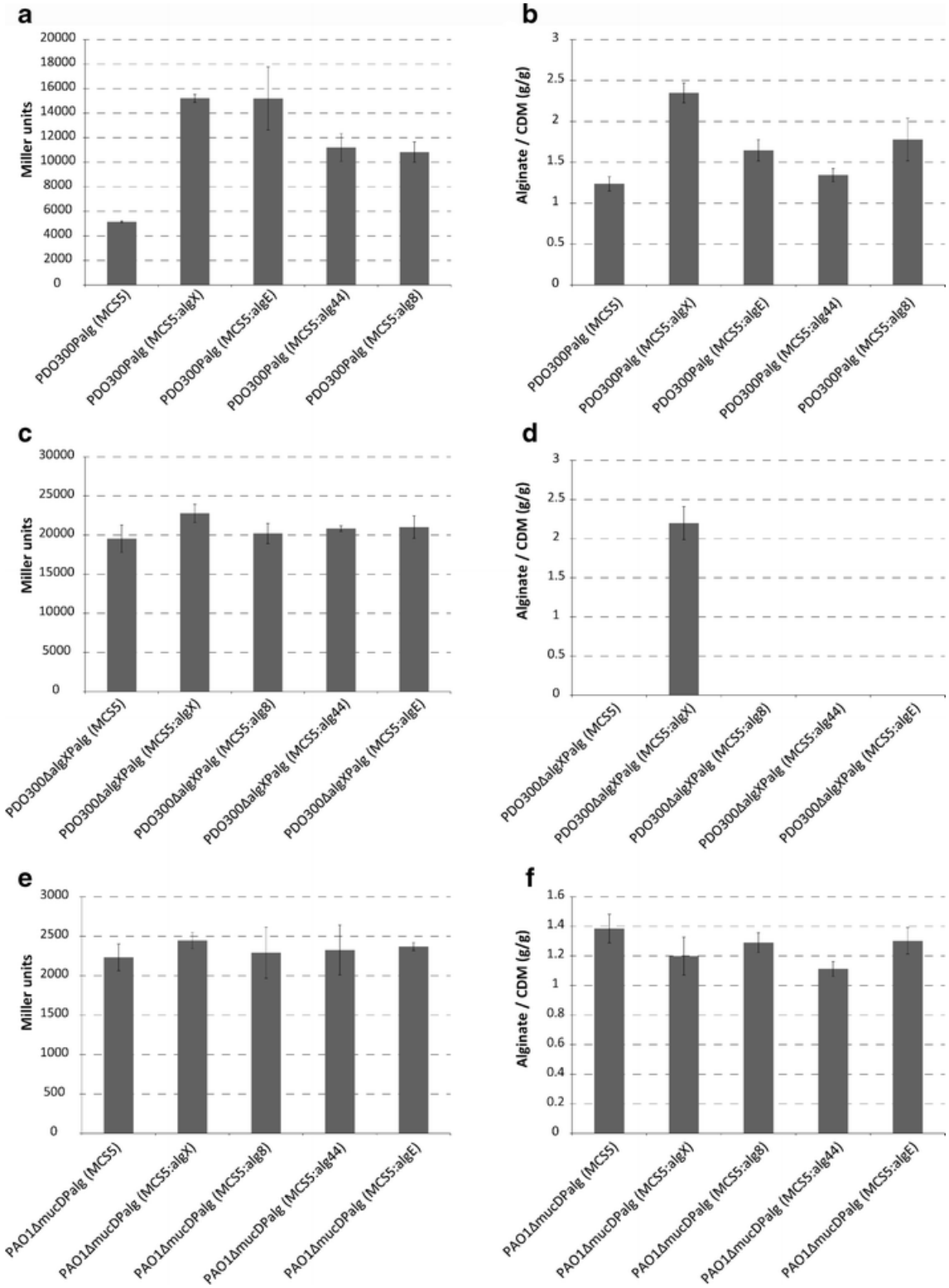

Figure 3: Alginate promoter activity (as measured by β-galactosidase activity) (**a**, **c**, **e** and **g**) and alginate production (**b**, **d**, **f** and **h)** of strains overproducing various proteins involved in alginate production. PDO300Palg is PDO300$_{CTXPalglacZ}$, PAO1Palg is PAO1$_{CTXPalglacZ}$, PDO300ΔalgXPalg is PDO300ΔalgX$_{CTXPalglacZ}$ and PAO1ΔmucDPalg is PAO1ΔmucD$_{CTXPalglacZ}$

Overexpression of *algX*, *algE*, *alg44* and *alg8* in PDO300$_{CTXPalglacZ}$ resulted in significant increases in transcription from the alginate promoter (3-, 3-, 2- and 2-fold, respectively) (Fig. 2a). Levels of alginate production in these strains did not tightly correlate with promoter activity, but all strains did produce elevated levels of alginate (Fig. 3b). Levels of dialyzable uronic acids were about 10-fold higher in PDO300 strains containing multiple copies of *algX*, *alg44* and *algE*, whereas dialyzable uronic acid levels were only slightly elevated when multiple copies of *alg8* were present (Supplementary Table 3). Overexpression of *algX*, *algE*, *alg44* or *alg8* in PAO1 neither induced transcription from the alginate promoter nor the production of alginate or dialyzable uronic acids (data not shown and Supplementary Table 3).

Overexpression of *algE*, *alg44* and *alg8* in PDO300ΔalgX resulted in no significant increases in transcription from the alginate promoter (when compared to PDO300ΔalgX). Complementation by overexpression of *algX* resulted in a slight increase in transcription levels (Fig. 3c). As expected, only expression of *algX* in PDO300ΔalgX could restore alginate production (Fig. 3d). Apart from the complemented strain, which showed 33% dialyzable uronic acid, all strains showed similar levels of uronic acids in the culture supernatant to PDO300ΔalgX(pBBR1MCS-5) with 100% of it being dialyzable (Supplementary Table 3).

Contrary to PDO300, overexpression of *algX*, *algE*, *alg44* and *alg8* in mucoid strain PAO1ΔmucD had no effect on the level of promoter activity or the level of alginate production (Fig. 2e, f). Free uronic acid constituted 9.91% of the total uronic acids in the culture supernatant of the PAO1ΔmucD(pBBR1MCS-5) strain and increased to 17.2%, 16.4% and 22.3% when multiple copies of *algX*, *alg44* or *algE*, respectively were present (Supplementary Table 3).

MucD can act both as a negative and positive regulator. As discussed above, disruption of *mucD* in PAO1 resulted in a mucoid phenotype and increased alginate promoter activity. Overexpression of mucD in PAO1 did not have an impact on the level of transcription from the alginate promoter act or on the levels of alginate production. Complementation of PAO1ΔmucD with *mucD* in *trans* resulted in a near complete reduction in promoter activity and alginate production (Fig. 4a, b). This does not seem to be mirrored in PDO300. Surprisingly, overexpression of *mucD* (thought to be a negative regulator) in PDO300 resulted in the most marked increase in promoter transcription observed (3.3-fold) (Fig. 4a, b). As the only difference between PDO300 and PAO1 is the

defective mucA22 allele, it would seem that MucD is playing a different, positive regulatory role in the absence of full-length MucA.

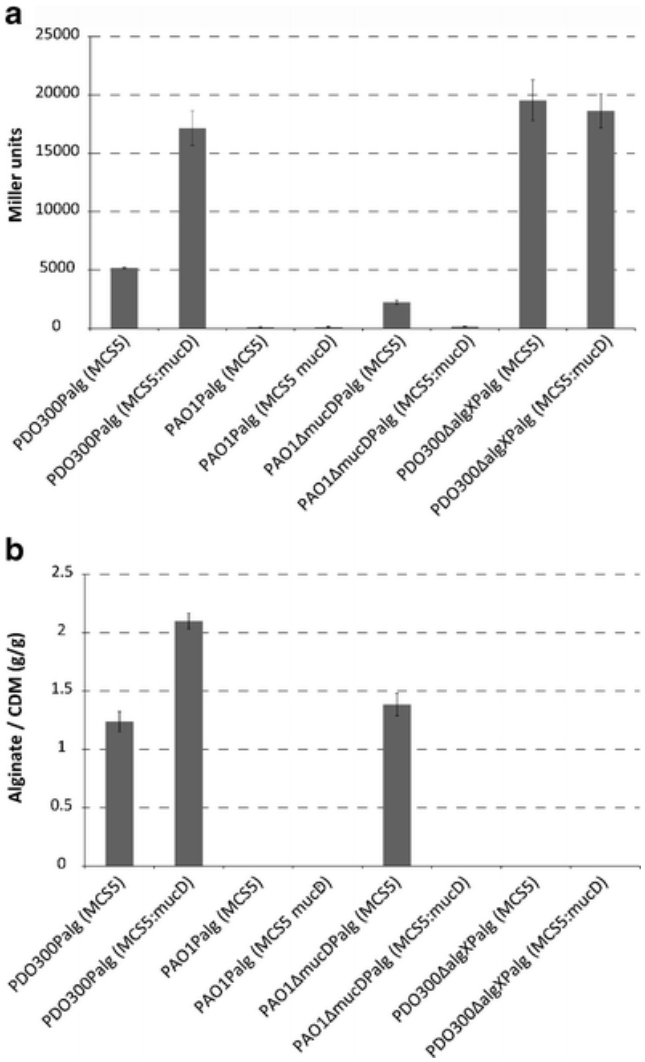

Figure 4: Alginate promoter activity (as measured by β-galactosidase activity) (**a**) and alginate production (**b**) strains overproducing MucD. PDO300Palg is $PDO300_{CTXPalglacZ}$, PAO1Palg is $PAO1_{CTXPalglacZ}$, PDO300ΔalgXPalg is $PDO300\Delta algX_{CTXPalglacZ}$ and PAO1ΔmucDPalg is $PAO1\Delta mucD_{CTXPalglacZ}$

Increased levels of transcription associated with aberrations to the stoichiometry of the alginate biosynthesis proteins require AlgU. Since PDO300 has a truncated (and seemingly non-functional) MucA, we hypothesised that changes in alginate promoter activity in PDO300-derived strains may be via a route independent of the conventional MucA–AlgU, anti-sigma factor, complex. Within the alginate promoter region, there is a binding site for an alternative sigma factor, RpoN. Under certain conditions, RpoN has been shown to be required for transcription from the alginate promoter, while inhibiting transcription under other conditions (15). Thus, the genes encoding the sigma factors AlgU and RpoN were disrupted in several strains to assess which sigma factor is responsible for the changes in the activation of the alginate promoter.

Disruption of *algU* in all strains assessed resulted in a loss of alginate production as well as reduction of alginate promoter activity to levels similar to those of PAO1, indicating that the increases in alginate promoter transcription observed in the ΔalgX and ΔmucD mutants ultimately require the AlgU sigma factor (Supplementary Table 4). Furthermore, overproduction AlgX, Alg8, Alg44, AlgE and MucD in PDO300ΔalgU did not result in increased levels of transcription seen in WT PDO300.

Disruption of *rpoN* produced a slight reduction in both alginate promoter activity and levels of alginate produced in PDO300. PAO1ΔmucDΔrpoN had no change in promoter activation nor in the levels of alginate produced. Artificially changing the levels of AlgX, Alg8, Alg44, AlgE or MucD had no significant effect on the increased levels of transcription associated with them (Supplementary Table 4).

Discussion

For decades, an alginate polymerisation/secretion multi-protein complex anchored in the cytoplasmic membrane had been proposed (Fig. 1) (127). So far, only one protein–protein interaction between AlgX and MucD had been experimentally shown (57). To provide experimental evidence for this multi-protein complex, it was investigated whether the AlgX–MucD complex interacts with further subunits. Here pull-down experiments using affinity chromatography were conducted. This led to identification of AlgK interacting with AlgX which interacts with MucD (Fig. 2). This is the first direct interaction demonstrated between essential alginate biosynthesis proteins. AlgK is a lipoprotein of unclear function which is encoded in the alginate operon and essential for the mucoid phenotype and the production of full-length alginate (75). AlgK is associated with the outer membrane, and there is some evidence to suggest that AlgK is involved in the localisation of AlgE to the outer membrane (81). When AlgX was purified from PDO300ΔalgX(pBBR1MCS-5:algXStrep), AlgK was predominately present in a truncated 45.6-kDa version with small amounts of full-length AlgK and MucD. However, when purified in the absence of MucD (i.e. from PAO1ΔmucDΔalgX(pBBR1MCS-5:algXStrep)), AlgK was only present in the full-length form. This could indicate that the proteolytic activity of MucD is cleaving AlgK, though the reason for this seems unclear. The fact MucD was present in far lower quantities than either AlgX or AlgK and that no MucD containing complex could be detected in the gel filtration chromatography suggested that the interaction between MucD and AlgX/AlgK is weak or transient. MucD could be co-purified with AlgX from the non-mucoid PAO1ΔalgX(pBBR1MCS-5:algXStrep), suggesting that the AlgX– MucD interaction is not dependent on AlgK. Similarly, co-purification of AlgK from the mucoid strain PAO1ΔmucDΔalgX(pBBR1MCS-5:algXStrep), i.e. in the absence of MucD, suggests that the AlgX–AlgK interaction is not dependent on MucD. Additionally, AlgX or AlgK does not appear to have an effect on the protease activity of MucD.

One possible explanation could be that the interaction of AlgX/AlgK with MucD might cause sequestration of MucD at the alginate polymerisation/secretion complex, making it unavailable for its regulatory role (Fig. 1). External stresses could cause instabilities in the complex and the release of MucD from the complex where it could exert its positive regulatory role increasing transcription of the alginate operon (possibly restoring the multi-protein complex).

The presence of the regulatory protein, MucD, in the periplasmic multi-protein complex suggested a link between the assembly of the multi-protein complex and regulation of

alginate biosynthesis. Regulation of alginate biosynthesis is a complex process involving a combination of transcriptional regulation, post-translational regulation and the mutation of "hyper-mutable" regions of the genome. This regulatory network involves both globally acting regulators and alginate-specific regulators. Recently, several of the steps of a RIP cascade involved in activating transcription of the alginate operon in response to cell wall stresses have been elucidated. At least five proteases have been shown to be involved in the proteolysis of MucA, and all but MucD have been shown to positively influence alginate production in non-mucod strains (122, 123, 180-182) (Fig. 1). MucD is thought to repress the activation of alginate production by degrading misfolded proteins that would otherwise activate the protease AlgW, though this relationship is unclear as it has recently been demonstrated that *mucD* mutants remain mucoid in the absence of AlgW but are dependent on the MucP protease (35, 123).

Here it was shown that there is a connection between the stability of the proposed alginate biosynthetic complex and the level of transcription from the alginate promoter. Removing or increasing the copies of various members of the proposed complex results in instability of the complex as indicated by the secretion of short dialysable uronic acids, presumably the products of alginate degradation by AlgL (138). In PDO300, overproduction of the various subunits resulted in an at least 2-fold increase in the level of transcription from the alginate promoter as well as increased levels of alginate biosynthesis (Fig. 2). The different levels of increased promoter activity observed with the different subunits could be due to the relative effect each subunit has on the stability of the complex. It appears that this activation is dependent on MucD, as overproduction of these same proteins in the mucoid PAO1ΔmucD had no significant effect on the levels of transcription from the alginate promoter or the levels of alginate production. It is possible that AlgX is also required as no increase in transcription was observed in PDO300ΔalgX strains overproducing these proteins, but any effect may be masked by the already elevated levels of transcription observed in this strain.

PDO300 is an isogenic derivative of PAO1 with a truncated, seemingly non-functional MucA anti-sigma factor (99), yet the increases in transcription associated with instability of the alginate biosynthesis complex appeared to be dependent on the sigma factor AlgU. This suggested that any communication between the alginate secretion complex and the transcriptional machinery still involves AlgU. As the truncation in MucA is in the periplasmic C-terminus, it is possible that the truncated MucA present in PDO300 (and many clinical isolates) is still able to bind/sequester the cytosolic AlgU, but may be more

prone to proteolysis by the RIP cascade. Instability of the secretion complex may speed up the proteolysis of MucA, possibly via MucD, and thus increase the levels of transcription. It should also be noted that instability of the complex (as assessed here) is not sufficient to induce expression of the alginate biosynthesis genes or a mucoid phenotype in the non-mucoid strain PAO1 containing full-length MucA.

Interestingly, though our results regarding the disruption of *mucD* in PAO1 are consistent with the proposed negative regulatory role of MucD, our results regarding the overproduction of MucD seem to suggest a positive regulatory role, at least in the absence of full-length MucA. Overproduction of MucD in PDO300 resulted in a 3.3-fold increase in the levels of transcription from the alginate promoter and a 1.7-fold increase in the levels of alginate biosynthesis (Fig. 3). This positive regulatory role would seem to be dependent on AlgX, as overproduction of MucD in PDO300ΔalgX does not result in an increased transcription, though this could be due to the already elevated transcription levels in this strain. Overproduction of MucD in PAO1 has neither effect on the levels of transcription nor the levels of alginate biosynthesis. This suggests that in the presence of full-length MucA, MucD acts as a negative regulator; however, in situations where MucA is truncated (i.e. PDO300), MucD may be acting as a positive regulator. This is complicated by the fact that it was not apparently possible to generate a *mucD*-deficient strain in the mucA22 strains PDO300 and FRD1. It cannot be ruled out that overproduction of MucD aids in the degradation of MucA when it is already truncated (and thus not "protected" by MucB) due to the muc22A mutation. Frame shift mutations in *mucA* resulting in truncations, such as the mucA22 mutation, are by far the most common mutations observed in clinical mucoid isolates (17, 30). This inability to generate the Δ*mucD* mutant could suggest that MucD may be essential for the survival in mucA22-based mucoid strains. This is strengthened by the finding that PAO1ΔmucD and PAO1ΔmucDΔalgX showed impaired growth and were prone to cell lysis. Also, Δ*mucD* mutants have previously been demonstrated to be more susceptible to heat and reactive oxygen species (14).

Intriguingly, though PAO1ΔmucD produced alginate at levels equivalent to PDO300, the alginate promoter activity was about 6.5 times less than in PDO300. This could indicate that the increase in alginate production observed with the loss of *mucD* may, at least to some extent, occur at a post-transcriptional level.

Overall, in this study, further evidence for a periplasmic multi-protein complex involved in alginate biosynthesis and its transcriptional regulation was obtained.

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Supplementary Material

Supplementary Table 1: Strains plasmids and oligonucleotides

Strain, Plasmid, or oligonucleotide	Description or sequence	Source or reference
P. aeruginosa		
PAO1	Prototrophic wild-type strain; Alg$^-$	(72)
PDO300	*muc22A* isogenic mutant derived from PAO1	(99)
PAO1ΔmucD	Isogenic *mucD* deletion mutant derived from PAO1	This study
PAO1ΔalgX	Isogenic *algX* deletion mutant derived from PAO1	This study
PAO1ΔmucDΔalgX	Isogenic *algX* deletion mutant derived from PAO1ΔmucD.	This study
PDO300ΔalgX	Isogenic *algX* deletion mutant derived from PDO300	This study
PDO300Δalg8	Isogenic *alg8* deletion mutant derived from PDO300	(137)
PDO300Δalg44	Isogenic *alg44* deletion mutant derived from PDO300	(135)
PDO300ΔalgE	Isogenic *algE* deletion mutant derived from PDO300	(66)
PAO1ΔalgU	Isogenic *algU* deletion mutant derived from PAO1	This study
PAO1ΔmucDΔalgU	Isogenic *algU* deletion mutant derived from PAO1ΔmucD 1	This study
PDO300ΔalgU	Isogenic *algU* deletion mutant derived from PDO300	This study
PDO300ΔalgXΔalgU	Isogenic *algU* deletion mutant derived from PDO300ΔalgX	This study
PAO1ΔrpoN	Isogenic *rpoN* deletion mutant derived from PAO1	This study
PAO1ΔmucDΔrpoN	Isogenic *rpoN* deletion mutant derived from PAO1ΔmucD	This study
PDO300ΔrpoN	Isogenic *rpoN* deletion mutant derived from PDO300	This study
PDO300ΔalgXΔrpoN	Isogenic *rpoN* deletion mutant derived from PDO300ΔalgX	This study
$PAO1_{CTXPalglacZ}$	PAO1 with *lacZ* under the control of the alginate promoter integrated into the genome at the *attB* site	This study
$PAO1\Delta mucD_{CTXPalglacZ}$	PAO1ΔmucD with *lacZ* under the control of the alginate promoter integrated into the genome at the *attB* site	This study
$PDO300_{CTXPalglacZ}$	PDO300 with *lacZ* under the control of the alginate promoter integrated into the genome at the *attB* site	This study
$PDO300\Delta algX_{CTXPalglacZ}$	PDO300ΔalgX with *lacZ* under the control of the alginate promoter integrated into the genome at the *attB* site	This study
$PDO300_{CTXPBADmucA}$	PDO300 with *mucA* under the control of the PBAD promoter integrated into the genome at the *attB* site	This study

E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
Rosetta 2	Has rare codons, AUA,, AGG, AGA, CUA, CCC, GGA, and CGG present on chloramphenicol resistant plasmid	Novagen
Plasmid		
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	(85)
pBBR1MCS-5:*mucD*	*mucD* inserted in to vector pBBR1MCS-5	This study
pBBR1MCS-5:*mucD*his	Translational MucD-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*algX*	*algX* inserted in to vector pBBR1MCS-5	(57)
pBBR1MCS-5:*algXStrep*	C-terminally Strep II tagged algX, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:*algXmucD*	*algX* and *mucD* inserted into vector pBBR1MCS-5	This study
mini-CTX-2	Self-proficient integration vector	(70)
mini-CTX-lacZ	Self-proficient integration vector, has promoterless lacZ	(9)
mini-CTX-2-PBADmucA	Self-proficient integration vector. Integrates *mucA* under the control of pBAD promoter	This study
mini-CTX-Palg-lacZ	Self-proficient integration vector, has lacZ under control of alginate promoter	This study
pEX100T	Ap^r Cb^r, gene replacement vector containing *sacB* gene for counterselection	(69)
pEX100TΔ*mucD*	Ap^r Cb^r Gm^r; vector pEX100T with SmaI-inserted *mucD* deletion construct	This study
pPS865	Ap^r Gm^r; source of 1,100-bp BamHI fragment comprising *aacC1* gene flanked by FRT signal sequences	(69)
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(69)
pTZ100	Promoterless lacZ on broad host range plasmid	(153)
pTZ100:Palg	lacZ under control of alginate promoter	(40)
Primers		
mucDF(HindIIISDNd)	GCCACGAAGCTTAGGAGTGTAGCATATGCATACCCTAAAACGCTGTATGGCTG	
mucDC6xHis(BamHI)	GTCTGGGATCCGACTTAATGATGGTGATGGTGGTGTTCGGCCAGCTTGAAGGTA ATGAAGCTGGCGCGTC	
mucAF(HindIIISDNd)	AGCGGAAGCTTAGGAGACGCTCATATGAGTCGTGAAGCCCTGCAGGAAACTCTG TCCG	
mucAR(BamHI)	GAGTAGGATCCGAGTCAGCGGTTTTCCAGGCTGGCTGCCCGAGC	
rpoN-NF-EcoRv	TGAACGATATCACTAAGCCCTCAGCCATGAAACCAT	
rpoN-NR-BamHI	TATAGGATCCGTGAAGTCCCACTCGTCATCGTCGT	
rpoN-CF-BamH	TACATGGATCCGTTTCCTCGACTACGGCGAAGAAGC	
rpoN-CR-EcoRV	CTTCAGATATCGGATCAACGTCACACCAGTCGCTTG	
algU-NF-ScaI	TCAATAGTACTTTTCCCCGTGGTGGAGCCCTAGTAT	
algU-NR-BamHI	TAAGTGGATCCTGATCCTGTTCCTGGGTTAGCATGA	
algU-CF-BamHI	ACTAGGATCCAGCCTTTGTTGCGAGAAGCCTGACA	
algU-CR-ScaI	TAGACAGTACTCCTTTCACCTGAGGCAGGGCGATCT	

Supplementary Table 2. Protease activity (as measured by release of resorufin from resorufin labelled casine) of MucD in the presence and absence of AlgX or other protein fractions that may influence the activity of MucD

	Resorufin released (nmol min)	
	Buffer	MucDHis (PAO1ΔmucDΔalgX)
Buffer	26.93 ± 2.91	76.99 ± 1.38
BSA	24.24 ± 2.51	60.16 ± 6.51
AlgXStrep (PAO1ΔmucDΔalgX)	39.50 ± 0.32	55.67 ± 1.77
AlgXStrep (Rosetta 2)	39.96 ± 2.821	56.34 ± 0.32
PAO1ΔmucDΔalgX (total membrane)	44.00 ± 1.93	59.48 ± 1.59
PAO1ΔmucD (total membrane)	46.24 ± 2.71	60.61 ± 1.10
PDO300ΔalgX (total membrane)	45.34 ± 4.80	61.05 ± 0.32
E. coli (total membrane)	44.66 ± 3.49	59.25 ± 4.70

Supplementary Table 3: % dialyzable free uronic acids of strains with multiple copies of proteins related to alginate biosynthesis

Strain	% dialyzable free uronic acids ± SD
PDO300 CTX_{Palg} (MCS5)	9.12 ± 2.65
PDO300 CTX_{Palg} (MCS5:algX)	32.86 ± 2.30
PDO300 CTX_{Palg} (MCS5:algE)	34.15 ± 9.03
PDO300 CTX_{Palg} (MCS5:alg44)	33.49 ± 3.87
PDO300 CTX_{Palg} (MCS5:alg8)	11.7 ± 7.32
PDO300ΔalgX CTX_{Palg} (MCS5)	100 ± 6.34
PDO300ΔalgX CTX_{Palg} (MCS5:algX)	33.14 ± 7.26
PDO300ΔalgX CTX_{Palg} (MCS5:alg8)	100 ± 5.25
PDO300ΔalgX CTX_{Palg} (MCS5:alg44)	100 ± 5.41
PDO300ΔalgX CTX_{Palg} (MCS5:algE)	100 ± 3.48
PAO1ΔmucD CTX_{Palg} (MCS5)	9.91 ± 3.55
PAO1ΔmucD CTX_{Palg} (MCS5:algX)	17.23 ± 2.761
PAO1ΔmucD CTX_{Palg} (MCS5:alg8)	12.93 ± 5.63
PAO1ΔmucD CTX_{Palg} (MCS5:alg44)	16.43 ± 3.72
PAO1ΔmucD CTX_{Palg} (MCS5:algE)	22.30 ± 3.60
PAO1ΔmucD CTX_{Palg} (MCS5)	9.91 ± 2.53
PAO1ΔmucD CTX_{Palg} (MCS5:mucDalgX)	7.36 ± 1.68
PAO1ΔmucDΔalgX CTX_{Palg} (MCS5)	100 ±4.24
PAO1ΔmucDΔalgX CTX_{Palg} (MCS5:mucD)	8.44 ± 1.06
PAO1ΔmucDΔalgX CTX_{Palg} (MCS5:algX)	16.64 ± 3.83
PAO1ΔmucDΔalgX CTX_{Palg} (MCS5:mucDalgX)	8.61 ± 3.16
PDO300ΔalgX CTX_{Palg} (MCS5:mucDalgX)	38.85 ± 3.66
PDO300 CTX_{Palg} (MCS5:mucD)	8.00 ±v 4.26
PAO1ΔmucD CTX_{Palg} (MCS5:mucD)	8.65 ± 3.78
PDO300ΔalgX CTX_{Palg} (MCS5:mucD)	100 ± 5.16

Supplementary Table 4: Promoter activity, alginate production and dialyzable uronic acids for AlgU and RpoN deficient strains

Strain	Alginate promoter Miller units ± SD	Alginate / CDW (g/g) ± SD	% dialyzable free uronic acids ± SD
PDO300 (pTZ110:Palg)	17811.71 ± 1177.65	1.91 ± 0.22	16.45 ±4.32
PDO300ΔalgU (pTZ110:Palg)	15.956 ± 13.18	ND	ND
PDO300ΔrpoN (pTZ110:Palg)	15028.22 ± 2096.34	1.77 ± 0.30	17.04 ± 10.56
PDO300ΔalgX (pTZ110:Palg)	38040.6 ± 4752.08	ND	100 ± 5.34
PDO300ΔalgXΔalgU (pTZ110:Palg)	19.18 ± 5.62	ND	ND
PDO300ΔalgXΔrpoN (pTZ110:Palg)	32299.14 ± 6623.72	ND	100 ± 9.63
PAO1 (pTZ110:Palg)	65.96 ± 6.42	ND	ND
PAO1ΔalgU (pTZ110:Palg)	20.83 ± 7.50	ND	ND
PAO1ΔrpoN (pTZ110:Palg)	78.11 ± 12.37	ND	ND
PAO1ΔmucD (pTZ110:Palg)	2784.76 ±584.85	1.54 ± 0.12	12.24 ± 5.28
PAO1ΔmucDΔalgU (pTZ110:Palg)	17.42 ± 4.19	ND	ND
PAO1ΔmucDΔrpoN (pTZ110:Palg)	2691.83 ± 892.91	ND	16.93 ± 5.84
PDO300 CTX_{Palg} (MCS5)	5150 ± 72.841	1.24 ± 0.09	-
PDO300 CTX_{Palg} (MCS5:algX)	15212.12 ± 321.652	2.35 ± 0.12	-
PDO300 CTX_{Palg} (MCS5:mucD)	17159.42 ± 1483.84	2.10 ± 0.07	-
PDO300 CTX_{Palg} (MCS5:algE)	15202.95 ± 2571.32	1.65 ± 0.13	-
PDO300 CTX_{Palg} (MCS5:alg44)	11209.38 ± 1132.04	1.34 ± 0.08	-
PDO300 CTX_{Palg} (MCS5:alg8)	10833.33 ± 832.42	1.78 ± 0.26	-
PDO300ΔalgU CTX_{Palg} (MCS5)	131.78 ± 11.28	ND	-
PDO300ΔalgU CTX_{Palg} (MCS5:algX)	156.67 ± 45.20	ND	-
PDO300ΔalgU CTX_{Palg} (MCS5:mucD)	59.76 ± 36.20	ND	-
PDO300ΔalgU CTX_{Palg} (MCS5:algE)	165.14 ± 36.60	ND	-
PDO300ΔalgU CTX_{Palg} (MCS5:alg44)	110.83 ± 21.83	ND	-
PDO300ΔalgU CTX_{Palg} (MCS5:alg8)	142.13 ± 72.80	ND	-
PDO300ΔrpoN CTX_{Palg} (MCS5)	5076.39 ± 409.87	1.00 ± 0.10	-
PDO300ΔrpoN CTX_{Palg} (MCS5:algX)	13059.70 ± 709.27	1.94 ± 0.22	-
PDO300ΔrpoN CTX_{Palg} (MCS5:mucD)	14610.73 ± 689.37	1.90 ± -.16	-
PDO300ΔrpoN CTX_{Palg} (MCS5:algE)	10738.58 ± 725.19	1.40 ± 0.09	-
PDO300ΔrpoN CTX_{Palg} (MCS5:alg44)	12263 ± 590.75	1.23 ± 0.24	-
PDO300ΔrpoN CTX_{Palg} (MCS5:alg8)	9770.83 ± 349.45	1.48 ± 0.11	-

SD - Standard deviation, ND - Not detected

Conclusions and Outlook

Conclusions

This thesis has provided insights in to both the regulation and the biosynthesis and secretion of alginate in *Pseudomonas aeruginosa*. A novel post transcriptional regulation of alginate biosynthesis was described. Loss of the inner membrane protein MucR protein with an undefined sensing domain (MHYT) and both a c-di-GMP cyclase domain (GGDEF) and phosphodiesterase domain resulted in diminished levels of alginate biosynthesis. This reduction in alginate biosynthesis appeared to be independent of other phenotypes generally associated with reduced levels of c-di-GMP. Furthermore, overproduction of another protein known to increase the total cellular levels of c-di-GMP did not significantly influence the levels of alginate biosynthesis. These results suggested to us that the MucR protein is specifically influencing alginate biosynthesis through modulating the levels of c-di-GMP which is presumably sensed by the c-di-GMP sensing domain of Alg44, which through an unknown mechanism, affects the levels of alginate polymerisation or secretion. It was proposed that this regulation is through the creation of a localised pool of c-di-GMP at the inner membrane.

Insights in to a complex periplasmic regulatory network were provided. Previously an interaction between an essential member of the alginate biosynthesis machinery (AlgX) and a member of the regulatory network involved in the regulation of the alginate operon (MucR) had been described. Here, this interaction was further investigated through assessing the activation of the alginate operon promoter in the absence or presence of increased levels of these and other related proteins. MucD has previously been proposed to act as a negative regulator through degrading misfolded proteins which would otherwise lead to the activation of the alginate promoter. We showed that in mucoid strains with mutations in *mucA* MucD appears to act as a positive regulator. It was also demonstrated that there is connection between the stability of the polymerisation / secretion complex and activation of the alginate promoter. Furthermore, the first direct interaction between members of the alginate biosynthesis / secretion machinery was demonstrated. Purification of AlgX lead to the co-purification of AlgK (another essential protein with unclear function).

The topology of the outer membrane porin AlgE was probed. It was proposed that the extended loop 7 folds down into the lumen of the pore and lines it. This loop was found to be essential for the folding and insertion of AlgE in to the outer membrane. Disruption of this loop by removal of this region or insertion of a FLAG tag in to this region inhibited insertion of the protein into the outer membrane.

Outlook

While the genes responsible for the biosynthesis of alginate have been known for two decades, the exact function of many of the encoded proteins remains unclear. Also unclear is how these proteins interact with one another. It has often been proposed that the membrane anchored and periplasmic proteins involved in alginate biosynthesis must form some kind of multi-protein complex, though no experimental evidence for this had been shown thus one of the key unanswered questions is how and if this multi-protein complex forms and functions.

Dr. P. Lynne Howell and co-workers at The Hospital for Sick Children at the University of Toronto have recently crystallized AlgE (177). Currently this group is resolving the structure of the protein. As part of an on-going collaboration, we are investigating, with respect to function, the roles of several regions of AlgE. The preliminary structure for AlgE has confirmed our prediction of the loop 7 region bending into the lumen of the pore (Howell PL personal communication) and has led to more informed targeting of regions and residues that may be involved in function.

There is some evidence to suggest that several of the other alginate biosynthesis proteins may interact with AlgE. Alg44 contains a membrane fusion domain in the periplasmic C-terminus which is commonly interacts with the outer membrane subunits of multi-drug efflux pumps and putative interacting domains have been recently identified in the structure of the periplasmic lipoprotein AlgK (81, 135). This taken together with the AlgK-AlgX interaction described in chapter V of this thesis, would indicate that with more thorough pull down experiments (using different detergents and buffers) may provide greater insights in to the polymerisation / secretion complex. Preliminary results obtained in by us and other labs (81) suggest that mutual stability experiments may provide insights in to any potential interactions occurring between alginate proteins.

The putative signal which may bind to the MHYT domain of the post translational regulator MucR needs to be further investigated, though it is unclear exactly how the signal would be identified. The three most obvious candidates appear to be CO, NO or O. Site directed mutagenesis of the poorly understood MHYT domains may aid in this process.

Though this thesis has provided some insights in to the interactions between the alginate biosynthetic machinery and those proteins which regulate the transcription of these

proteins, it is still unclear exactly how and why this connection functions. One of the key unresolved questions is what MucD is doing in mucoid strains. The overproduction of MucD would seem to suggest that it is positively influencing transcription whereas knocking MucD out in the non mucoid strain also positively influences transcription.

References

References

1. **Aarons, S. J., I. W. Sutherland, A. M. Chakrabarty, and M. P. Gallagher.** 1997. A novel gene, algK, from the alginate biosynthesis cluster of *Pseudomonas aeruginosa*. Microbiology **143 (Pt 2):**641-52.

2. **Albrecht, M. T., and N. L. Schiller.** 2005. Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol **187:**3869-72.

3. **Amikam, D., and M. Y. Galperin.** 2006. PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics **22:**3-6.

4. **Anantharaman, V., and L. Aravind.** 2003. Application of comparative genomics in the identification and analysis of novel families of membrane-associated receptors in bacteria. BMC Genomics **4:**34.

5. **Bagos, P. G., T. D. Liakopoulos, I. C. Spyropoulos, and S. J. Hamodrakas.** 2004. PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins. Nucleic Acids Res **32:**W400-4.

6. **Bakkevig, K., H. Sletta, M. Gimmestad, R. Aune, H. Ertesvag, K. Degnes, B. E. Christensen, T. E. Ellingsen, and S. Valla.** 2005. Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment. J Bacteriol **187:**8375-84.

7. **Baynham, P. J., A. L. Brown, L. L. Hall, and D. J. Wozniak.** 1999. *Pseudomonas aeruginosa* AlgZ, a ribbon-helix-helix DNA-binding protein, is essential for alginate synthesis and *algD* transcriptional activation. Mol Microbiol **33:**1069-80.

8. **Baynham, P. J., and D. J. Wozniak.** 1996. Identification and characterization of AlgZ, an AlgT-dependent DNA-binding protein required for *Pseudomonas aeruginosa algD* transcription. Mol Microbiol **22:**97-108.

9. **Becher, A., and H. P. Schweizer.** 2000. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. Biotechniques **29:**948-50, 952.

10. **Bieber Urbauer, R. J., J. M. Gilmore, S. E. Rosasco, J. M. Hattle, A. B. Cowley, and J. L. Urbauer.** 2005. Cloning, high yield overexpression, purification, and characterization of AlgH, a regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Protein Expr Purif **43:**57-64.

11. **Blatch, G. L., and M. Lassle.** 1999. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays **21:**932-9.

12. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal Biochem **54:**484-9.

13. **Bornet, C., N. Saint, L. Fetnaci, M. Dupont, A. Davin-Regli, C. Bollet, and J. M. Pages.** 2004. Omp35, a new *Enterobacter aerogenes* porin involved in selective susceptibility to cephalosporins. Antimicrob Agents Chemother **48:**2153-8.

14. **Boucher, J. C., J. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. J Bacteriol **178:**511-23.

15. **Boucher, J. C., M. J. Schurr, and V. Deretic.** 2000. Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. Mol Microbiol **36:**341-51.

16. **Boucher, J. C., M. J. Schurr, H. Yu, D. W. Rowen, and V. Deretic.** 1997. *Pseudomonas aeruginosa* in cystic fibrosis: role of *mucC* in the regulation of alginate production and stress sensitivity. Microbiology **143 (Pt 11):**3473-80.

17. **Boucher, J. C., H. Yu, M. H. Mudd, and V. Deretic.** 1997. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of *muc* mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect Immun **65:**3838-46.

18. **Boyd, A., and A. M. Chakrabarty.** 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl Environ Microbiol **60:**2355-9.

19. **Boyd, A., M. Ghosh, T. B. May, D. Shinabarger, R. Keogh, and A. M. Chakrabarty.** 1993. Sequence of the *algL* gene of *Pseudomonas aeruginosa* and purification of its alginate lyase product. Gene **131:**1-8.

20. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248-54.

21. **Bragonzi, A., D. Worlitzsch, G. B. Pier, P. Timpert, M. Ulrich, M. Hentzer, J. B. Andersen, M. Givskov, M. Conese, and G. Doring.** 2005. Nonmucoid *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. J Infect Dis **192:**410-9.

22. **Campisano, A., C. Schroeder, M. Schemionek, J. Overhage, and B. H. Rehm.** 2006. PslD is a secreted protein required for biofilm formation by *Pseudomonas aeruginosa*. Appl Environ Microbiol **72:**3066-8.

23. **Cezairliyan, B. O., and R. T. Sauer.** 2009. Control of *Pseudomonas aeruginosa* AlgW protease cleavage of MucA by peptide signals and MucB. Mol Microbiol **72:**368-79.

24. **Chitnis, C. E., and D. E. Ohman.** 1990. Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J Bacteriol **172:**2894-900.

25. **Chitnis, C. E., and D. E. Ohman.** 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol Microbiol **8:**583-93.

26. **Chiu, J., D. Tillett, I. W. Dawes, and P. E. March.** 2008. Site-directed, Ligase-Independent Mutagenesis (SLIM) for highly efficient mutagenesis of plasmids greater than 8kb. J Microbiol Methods **73:**195-8.

27. **Choi, K. H., A. Kumar, and H. P. Schweizer.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods **64:**391-7.

28. **Choy, W. K., L. Zhou, C. K. Syn, L. H. Zhang, and S. Swarup.** 2004. MorA defines a new class of regulators affecting flagellar development and biofilm formation in diverse *Pseudomonas* species. J Bacteriol **186:**7221-8.

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

30. **Ciofu, O., B. Lee, M. Johannesson, N. O. Hermansen, P. Meyer, and N. Hoiby.** 2008. Investigation of the *algT* operon sequence in mucoid and non-mucoid *Pseudomonas aeruginosa* isolates from 115 Scandinavian patients with cystic fibrosis and in 88 in vitro non-mucoid revertants. Microbiology **154:**103-13.

31. **Costerton, J. W.** 2001. Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. Trends Microbiol **9:**50-2.

32. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318-22.

33. **D'Andrea, L. D., and L. Regan.** 2003. TPR proteins: the versatile helix. Trends Biochem Sci **28:**655-62.

34. **D'Argenio, D. A., M. W. Calfee, P. B. Rainey, and E. C. Pesci.** 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. J Bacteriol **184:**6481-9.

35. **Damron, F. H., and H. D. Yu.** 2011. *Pseudomonas aeruginosa* MucD regulates the alginate pathway through activation of MucA degradation via MucP proteolytic activity. J Bacteriol **193:**286-91.

36. **Darzins, A., and A. M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J Bacteriol **159:**9-18.

37. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J Bacteriol **176:**2773-80.

38. **Elkins, C. A., and H. Nikaido.** 2003. Chimeric analysis of AcrA function reveals the importance of its C-terminal domain in its interaction with the AcrB multidrug efflux pump. J Bacteriol **185:**5349-56.

39. **Ertesvag, H., H. K. Hoidal, H. Schjerven, B. I. Svanem, and S. Valla.** 1999. Mannuronan C-5-epimerases and their application for in vitro and in vivo design of new alginates useful in biotechnology. Metab Eng **1:**262-9.

40. **Ethier, J., and J. M. Boyd.** 2000. Topological analysis and role of the transmembrane domain in polar targeting of PilS, a *Pseudomonas aeruginosa* sensor kinase. Mol Microbiol **38:**891-903.

41. **Finelli, A., C. V. Gallant, K. Jarvi, and L. L. Burrows.** 2003. Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. J Bacteriol **185:**2700-10.

42. **Finn, R. D., J. Mistry, B. Schuster-Bockler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon, M. Marshall, A. Khanna, R. Durbin, S. R. Eddy, E. L. Sonnhammer, and A. Bateman.** 2006. Pfam: clans, web tools and services. Nucleic Acids Res **34:**D247-51.

43. **Finn, R. D., J. Tate, J. Mistry, P. C. Coggill, S. J. Sammut, H. R. Hotz, G. Ceric, K. Forslund, S. R. Eddy, E. L. Sonnhammer, and A. Bateman.** 2008. The Pfam protein families database. Nucleic Acids Res **36:**D281-8.

44. **Firoved, A. M., J. C. Boucher, and V. Deretic.** 2002. Global genomic analysis of AlgU (sigma(E))-dependent promoters (sigmulon) in *Pseudomonas aeruginosa* and implications for inflammatory processes in cystic fibrosis. J Bacteriol **184:**1057-64.

45. **Firoved, A. M., and V. Deretic.** 2003. Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. J Bacteriol **185:**1071-81.

46. **Firoved, A. M., S. R. Wood, W. Ornatowski, V. Deretic, and G. S. Timmins.** 2004. Microarray analysis and functional characterization of the nitrosative stress response in nonmucoid and mucoid *Pseudomonas aeruginosa*. J Bacteriol **186:**4046-50.

47. **Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman.** 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. J Bacteriol **176:**1821-30.

48. **Franklin, M. J., S. A. Douthit, and M. A. McClure.** 2004. Evidence that the *algI/algJ* gene cassette, required for O acetylation of *Pseudomonas aeruginosa* alginate, evolved by lateral gene transfer. J Bacteriol **186:**4759-73.

49. **Franklin, M. J., and D. E. Ohman.** 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J Bacteriol **175:**5057-65.

50. **Franklin, M. J., and D. E. Ohman.** 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. J Bacteriol **184:**3000-7.

51. **Galperin, M. Y., T. A. Gaidenko, A. Y. Mulkidjanian, M. Nakano, and C. W. Price.** 2001. MHYT, a new integral membrane sensor domain. FEMS Microbiol Lett **205:**17-23.

52. **Gimmestad, M., H. Ertesvag, T. M. Heggeset, O. Aarstad, B. I. Svanem, and S. Valla.** 2009. Characterization of three new *Azotobacter vinelandii* alginate lyases, one of which is involved in cyst germination. J Bacteriol **191:**4845-53.

53. **Gimmestad, M., H. Sletta, H. Ertesvag, K. Bakkevig, S. Jain, S. J. Suh, G. Skjak-Braek, T. E. Ellingsen, D. E. Ohman, and S. Valla.** 2003. The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J Bacteriol **185:**3515-23.

54. **Goldberg, J. B., K. Hatano, and G. B. Pier.** 1993. Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. J Bacteriol **175:**1605-11.

55. **Grabert, E., J. Wingender, and U. K. Winkler.** 1990. An outer membrane protein characteristic of mucoid strains of *Pseudomonas aeruginosa*. FEMS Microbiol Lett **56:**83-7.

56. **Gutierrez, C., and J. C. Devedjian.** 1989. A plasmid facilitating in vitro construction of *phoA* gene fusions in *Escherichia coli*. Nucleic Acids Res **17:**3999.

57. **Gutsche, J., U. Remminghorst, and B. H. Rehm.** 2006. Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie **88:**245-51.

58. **Guvener, Z. T., and C. S. Harwood.** 2007. Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. Mol Microbiol **66:**1459-73.

59. **Hancock, R. E., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- 2-mercaptoethanol-modifiable proteins. J Bacteriol **140:**902-10.

60. **Hasse, C., G. Klock, A. Schlosser, U. Zimmermann, and M. Rothmund.** 1997. Parathyroid allotransplantation without immunosuppression. Lancet **350:**1296-7.

61. **Hasse, C., A. Zielke, G. Klock, P. Barth, A. Schlosser, U. Zimmermann, and M. Rothmund.** 1997. First successful xenotransplantation of microencapsulated human parathyroid tissue in experimental hypoparathyroidism: long-term function without immunosuppression. J Microencapsul **14:**617-26.

62. **Hasse, C., A. Zielke, G. Klock, A. Schlosser, P. Barth, U. Zimmermann, H. Sitter, W. Lorenz, and M. Rothmund.** 1998. Amitogenic alginates: key to first clinical application of microencapsulation technology. World J Surg **22:**659-65.

63. **Hasse, C., A. Zielke, G. Klock, A. Schlosser, U. Zimmermann, and M. Rothmund.** 1997. Isotransplantation of microencapsulated parathyroid tissue in rats. Exp Clin Endocrinol Diabetes **105:**53-6.

64. **Haussler, S.** 2004. Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. Environ Microbiol **6:**546-51.

65. **Hay, I. D., K. Gatland, A. Campisano, J. Z. Jordens, and B. H. Rehm.** 2009. Impact of alginate overproduction on attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa* strain. Appl Environ Microbiol **75:**6022-5.

66. **Hay, I. D., Z. U. Rehman, and B. H. Rehm.** 2010. Membrane topology of outer membrane protein AlgE, which is required for alginate production in *Pseudomonas aeruginosa*. Appl Environ Microbiol **76:**1806-12.

67. **Hay, I. D., U. Remminghorst, and B. H. Rehm.** 2009. MucR, a novel membrane-associated regulator of alginate biosynthesis in *Pseudomonas aeruginosa*. Appl Environ Microbiol **75:**1110-20.

68. **Hay, I. D., Z. Ur Rehman, A. Ghafoor, and B. H. A. Rehm.** 2010. Bacterial biosynthesis of alginates. Journal of Chemical Technology & Biotechnology **85:**752-759.

69. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.

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

71. **Hoidal, H. K., B. I. Glaerum Svanem, M. Gimmestad, and S. Valla.** 2000. Mannuronan C-5 epimerases and cellular differentiation of *Azotobacter vinelandii*. Environ Microbiol **2:**27-38.

72. **Holloway, B. W., H. Matsumoto, and P. V. Phibbs, Jr.** 1986. The chromosome map of Pseudomonas aeruginosa PAO. Acta Microbiol Pol **35:**161-4.

73. **Jackson, K. D., M. Starkey, S. Kremer, M. R. Parsek, and D. J. Wozniak.** 2004. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J Bacteriol **186:**4466-75.

74. **Jain, S., M. J. Franklin, H. Ertesvag, S. Valla, and D. E. Ohman.** 2003. The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol Microbiol **47:**1123-33.

75. **Jain, S., and D. E. Ohman.** 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J Bacteriol **180:**634-41.

76. **Jain, S., and D. E. Ohman.** 2005. Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infect Immun **73:**6429-36.

77. **Jap, B. K., P. J. Walian, and K. Gehring.** 1991. Structural architecture of an outer membrane channel as determined by electron crystallography. Nature **350:**167-70.

78. **Kato, J., L. Chu, K. Kitano, J. D. DeVault, K. Kimbara, A. M. Chakrabarty, and T. K. Misra.** 1989. Nucleotide sequence of a regulatory region controlling alginate synthesis in *Pseudomonas aeruginosa*: characterization of the *algR2* gene. Gene **84:**31-8.

79. **Kato, J., T. K. Misra, and A. M. Chakrabarty.** 1990. AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **87:**2887-91.

80. **Kazmierczak, B. I., M. B. Lebron, and T. S. Murray.** 2006. Analysis of FimX, a phosphodiesterase that governs twitching motility in *Pseudomonas aeruginosa*. Mol Microbiol **60:**1026-43.

81. **Keiski, C. L., M. Harwich, S. Jain, A. M. Neculai, P. Yip, H. Robinson, J. C. Whitney, L. Riley, L. L. Burrows, D. E. Ohman, and P. L. Howell.** 2010. AlgK is a TPR-containing protein and the periplasmic component of a novel exopolysaccharide secretin. Structure **18:**265-73.

82. **Klock, G., H. Frank, R. Houben, T. Zekorn, A. Horcher, U. Siebers, M. Wohrle, K. Federlin, and U. Zimmermann.** 1994. Production of purified alginates suitable for use in immunoisolated transplantation. Appl Microbiol Biotechnol **40:**638-43.

83. **Klock, G., A. Pfeffermann, C. Ryser, P. Grohn, B. Kuttler, H. J. Hahn, and U. Zimmermann.** 1997. Biocompatibility of mannuronic acid-rich alginates. Biomaterials **18:**707-13.

84. **Kong, H. J., E. Wong, and D. J. Mooney.** 2003. Independent Control of Rigidity and Toughness of Polymeric Hydrogels. Macromolecules **36:**4582-4588.

85. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, 2nd, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-6.

86. **Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol **305:**567-80.

87. **Kuchma, S. L., K. M. Brothers, J. H. Merritt, N. T. Liberati, F. M. Ausubel, and G. A. O'Toole.** 2007. BifA, a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by *Pseudomonas aeruginosa* PA14. J Bacteriol **189:**8165-78.

88. **Kulasakara, H., V. Lee, A. Brencic, N. Liberati, J. Urbach, S. Miyata, D. G. Lee, A. N. Neely, M. Hyodo, Y. Hayakawa, F. M. Ausubel, and S. Lory.** 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proc Natl Acad Sci U S A **103:**2839-44.

89. **Kulasekara, H. D., I. Ventre, B. R. Kulasekara, A. Lazdunski, A. Filloux, and S. Lory.** 2005. A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial *cup* genes. Mol Microbiol **55:**368-80.

90. **Letunic, I., R. R. Copley, B. Pils, S. Pinkert, J. Schultz, and P. Bork.** 2006. SMART 5: domains in the context of genomes and networks. Nucleic Acids Res **34:**D257-60.

91. **Lewenza, S., J. L. Gardy, F. S. Brinkman, and R. E. Hancock.** 2005. Genome-wide identification of *Pseudomonas aeruginosa* exported proteins using a consensus computational strategy combined with a laboratory-based PhoA fusion screen. Genome Res **15:**321-9.

92. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin Microbiol Rev **15:**194-222.

93. **Lynn, A. R., and J. R. Sokatch.** 1984. Incorporation of isotope from specifically labeled glucose into alginates of *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. J Bacteriol **158:**1161-2.

94. **Ma, S., U. Selvaraj, D. E. Ohman, R. Quarless, D. J. Hassett, and D. J. Wozniak.** 1998. Phosphorylation-independent activity of the response regulators AlgB and AlgR in promoting alginate biosynthesis in mucoid *Pseudomonas aeruginosa*. J Bacteriol **180:**956-68.

95. **Mai, G. T., J. G. McCormack, W. K. Seow, G. B. Pier, L. A. Jackson, and Y. H. Thong.** 1993. Inhibition of adherence of mucoid *Pseudomonas aeruginosa* by alginase, specific monoclonal antibodies, and antibiotics. Infect Immun **61:**4338-43.

96. **Manoil, C.** 1991. Analysis of membrane protein topology using alkaline phosphatase and beta-galactosidase gene fusions. Methods Cell Biol **34:**61-75.

97. **Martin, D. W., M. J. Schurr, M. H. Mudd, and V. Deretic.** 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: inactivation of *mucB* causes conversion to mucoidy. Mol Microbiol **9:**497-506.

98. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc Natl Acad Sci U S A **90:**8377-81.

99. **Mathee, K., O. Ciofu, C. Sternberg, P. W. Lindum, J. I. Campbell, P. Jensen, A. H. Johnsen, M. Givskov, D. E. Ohman, S. Molin, N. Hoiby, and A. Kharazmi.** 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiology **145 (Pt 6):**1349-57.

100. **Mathee, K., C. J. McPherson, and D. E. Ohman.** 1997. Posttranslational control of the *algT* (*algU*)-encoded sigma22 for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its antagonist proteins MucA and MucB (AlgN). J Bacteriol **179:**3711-20.

101. **May, T. B., D. Shinabarger, A. Boyd, and A. M. Chakrabarty.** 1994. Identification of amino acid residues involved in the activity of phosphomannose isomerase-guanosine 5'-diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J Biol Chem **269:**4872-7.

102. **Merighi, M., V. T. Lee, M. Hyodo, Y. Hayakawa, and S. Lory.** 2007. The second messenger bis-(3'-5')-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. Mol Microbiol **65:**876-95.

103. **Merritt, J. H., K. M. Brothers, S. L. Kuchma, and G. A. O'Toole.** 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. J Bacteriol **189:**8154-64.

104. **Merritt, J. H., D. E. Kadouri, and G. A. O'Toole.** 2005. Growing and analyzing static biofilms. Curr Protoc Microbiol **Chapter 1:**Unit 1B 1.

105. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, NY.

106. **Monday, S. R., and N. L. Schiller.** 1996. Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX. J Bacteriol **178:**625-32.

107. **Morch, Y. A., I. Donati, B. L. Strand, and G. Skjak-Braek.** 2007. Molecular engineering as an approach to design new functional properties of alginate. Biomacromolecules **8:**2809-14.

108. **Morch, Y. A., S. Holtan, I. Donati, B. L. Strand, and G. Skjak-Braek.** 2008. Mechanical properties of C-5 epimerized alginates. Biomacromolecules **9:**2360-8.

109. **Mougel, C., and I. B. Zhulin.** 2001. CHASE: an extracellular sensing domain common to transmembrane receptors from prokaryotes, lower eukaryotes and plants. Trends Biochem Sci **26:**582-4.

110. **Murray, T. S., and B. I. Kazmierczak.** 2006. FlhF is required for swimming and swarming in *Pseudomonas aeruginosa*. J Bacteriol **188:**6995-7004.

111. **Narbad, A., N. J. Russell, and P. Gacesa.** 1988. Radiolabelling patterns in alginate of *Pseudomonas aeruginosa* synthesized from specifically-labelled 14C-monosaccharide precursors. Microbios **54:**171-9.

112. **Nguyen, K. A., J. Zylicz, P. Szczesny, A. Sroka, N. Hunter, and J. Potempa.** 2009. Verification of a topology model of PorT as an integral outer-membrane protein in *Porphyromonas gingivalis*. Microbiology **155:**328-37.

113. **Nivens, D. E., D. E. Ohman, J. Williams, and M. J. Franklin.** 2001. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J Bacteriol **183:**1047-57.

114. **Oglesby, L. L., S. Jain, and D. E. Ohman.** 2008. Membrane topology and roles of *Pseudomonas aeruginosa* Alg8 and Alg44 in alginate polymerization. Microbiology **154:**1605-15.

115. **Olvera, C., J. B. Goldberg, R. Sanchez, and G. Soberon-Chavez.** 1999. The *Pseudomonas aeruginosa algC* gene product participates in rhamnolipid biosynthesis. FEMS Microbiol Lett **179:**85-90.

116. **Overhage, J., M. Schemionek, J. S. Webb, and B. H. Rehm.** 2005. Expression of the psl operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation. Appl Environ Microbiol **71:**4407-13.

117. **Paul, R., S. Weiser, N. C. Amiot, C. Chan, T. Schirmer, B. Giese, and U. Jenal.** 2004. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev **18:**715-27.

118. **Pedersen, S. S., N. Hoiby, F. Espersen, and C. Koch.** 1992. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax **47:**6-13.

119. **Pier, G. B., F. Coleman, M. Grout, M. Franklin, and D. E. Ohman.** 2001. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. Infect Immun **69:**1895-901.

120. **Pindar, D. F., and C. Bucke.** 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. Biochem J **152:**617-22.

121. **Qiu, D., F. H. Damron, T. Mima, H. P. Schweizer, and H. D. Yu.** 2008. PBAD-based shuttle vectors for functional analysis of toxic and highly regulated genes in *Pseudomonas* and *Burkholderia spp*. and other bacteria. Appl Environ Microbiol **74:**7422-6.

122. **Qiu, D., V. M. Eisinger, N. E. Head, G. B. Pier, and H. D. Yu.** 2008. ClpXP proteases positively regulate alginate overexpression and mucoid conversion in *Pseudomonas aeruginosa*. Microbiology **154:**2119-30.

123. **Qiu, D., V. M. Eisinger, D. W. Rowen, and H. D. Yu.** 2007. Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **104:**8107-12.

124. **Ramsey, D. M., and D. J. Wozniak.** 2005. Understanding the control of *Pseudomonas aeruginosa* alginate synthesis and the prospects for management of chronic infections in cystic fibrosis. Mol Microbiol **56:**309-22.

125. **Rehm, B. H.** 2002. Alginates from Bacteria, p. 179-211. *In* E. J. Vandamme, S. DeBaets, and A. Steinbuchel (ed.), Biopolymers, vol. Polysaccharides. Wiley VCH.

126. **Rehm, B. H.** 1996. The *Azotobacter vinelandii* gene *algJ* encodes an outer-membrane protein presumably involved in export of alginate. Microbiology **142 (Pt 4):**873-80.

127. **Rehm, B. H.** 2010. Bacterial polymers: biosynthesis, modifications and applications. Nat Rev Microbiol **8:**578-92.

128. **Rehm, B. H., G. Boheim, J. Tommassen, and U. K. Winkler.** 1994. Overexpression of *algE* in *Escherichia coli*: subcellular localization, purification, and ion channel properties. J Bacteriol **176:**5639-47.

129. **Rehm, B. H., H. Ertesvag, and S. Valla.** 1996. A new *Azotobacter vinelandii* mannuronan C-5-epimerase gene (*algG*) is part of an alg gene cluster physically organized in a manner similar to that in *Pseudomonas aeruginosa*. J Bacteriol **178:**5884-9.

130. **Rehm, B. H., E. Grabert, J. Hein, and U. K. Winkler.** 1994. Antibody response of rabbits and cystic fibrosis patients to an alginate-specific outer membrane protein of a mucoid strain of *Pseudomonas aeruginosa*. Microb Pathog **16:**43-51.

131. **Rehm, B. H., and R. E. Hancock.** 1996. Membrane topology of the outer membrane protein OprH from *Pseudomonas aeruginosa*: PCR-mediated site-directed insertion and deletion mutagenesis. J Bacteriol **178:**3346-9.

132. **Rehm, B. H., and S. Valla.** 1997. Bacterial alginates: biosynthesis and applications. Appl Microbiol Biotechnol **48:**281-8.

133. **Rehm, B. H. A.** 2005. Biosynthesis and applications of Alginates, p. 1-9. *In* G. Wnek and G. Bowlin (ed.), Encyclopedia of Biomaterials and Biomedical Engineering. Marcel Dekker Inc.

134. **Remminghorst, U., I. D. Hay, and B. H. Rehm.** 2009. Molecular characterization of Alg8, a putative glycosyltransferase, involved in alginate polymerisation. J Biotechnol **140:**176-83.

135. **Remminghorst, U., and B. H. Rehm.** 2006. Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. FEBS Lett **580:**3883-8.

136. **Remminghorst, U., and B. H. Rehm.** 2006. Bacterial alginates: from biosynthesis to applications. Biotechnol Lett **28:**1701-12.

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

138. **Robles-Price, A., T. Y. Wong, H. Sletta, S. Valla, and N. L. Schiller.** 2004. AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol **186:**7369-77.

139. **Romling, U., and D. Amikam.** 2006. Cyclic di-GMP as a second messenger. Curr Opin Microbiol **9:**218-28.

140. **Romling, U., M. Gomelsky, and M. Y. Galperin.** 2005. C-di-GMP: the dawning of a novel bacterial signalling system. Mol Microbiol **57:**629-39.

141. **Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. de Vroom, G. A. van der Marel, J. H. van Boom, and M. Benziman.** 1987. Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature **325:**279-81.

142. **Rowley, G., M. Spector, J. Kormanec, and M. Roberts.** 2006. Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. Nat Rev Microbiol **4:**383-94.

143. **Roychoudhury, S., T. B. May, J. F. Gill, S. K. Singh, D. S. Feingold, and A. M. Chakrabarty.** 1989. Purification and characterization of guanosine diphospho-D-mannose dehydrogenase. A key enzyme in the biosynthesis of alginate by *Pseudomonas aeruginosa*. J Biol Chem **264:**9380-5.

144. **Sabra, W., and A. Ping Zeng.** 2009. Microbial Production of Alginates: Physiology and Process Aspects. *In* B. H. A. Rehm (ed.), Alginates: Biology and Applications, vol. 13. Springer-Verlag, Berlin Heidelberg.

145. **Salzig, M., and B. H. A. Rehm.** 2006. Microbial Production of Alginates: Self Assembly and Applications, p. 125-152. *In* B. H. A. Rehm (ed.), Microbial Biotechnology: Biological self-assembly Systems and Biopolymer-based Nanostructures. Horizon Scientific Press, Norfolk, UK.

146. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

147. **Saxena, I. M., R. M. Brown, Jr., and T. Dandekar.** 2001. Structure--function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57:**1135-48.

148. **Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia, and B. Henrissat.** 1995. Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. J Bacteriol **177:**1419-24.

149. **Schiller, N. L., S. R. Monday, C. M. Boyd, N. T. Keen, and D. E. Ohman.** 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing, and expression in *Escherichia coli*. J Bacteriol **175:**4780-9.

150. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies]. Arch Mikrobiol **38:**209-22.

151. **Schurr, M. J., D. W. Martin, M. H. Mudd, N. S. Hibler, J. C. Boucher, and V. Deretic.** 1993. The *algD* promoter: regulation of alginate production by *Pseudomonas aeruginosa* in cystic fibrosis. Cell Mol Biol Res **39:**371-6.

152. **Schurr, M. J., H. Yu, J. M. Martinez-Salazar, J. C. Boucher, and V. Deretic.** 1996. Control of AlgU, a member of the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. J Bacteriol **178:**4997-5004.

153. **Schweizer, H. P., and R. Chuanchuen.** 2001. Small broad-host-range *lacZ* operon fusion vector with low background activity. Biotechniques **31:**1258, 1260, 1262.

154. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:**15-22.

155. **Shankar, S., R. W. Ye, D. Schlictman, and A. M. Chakrabarty.** 1995. Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. Adv Enzymol Relat Areas Mol Biol **70:**221-55.

156. **Shinabarger, D., A. Berry, T. B. May, R. Rothmel, A. Fialho, and A. M. Chakrabarty.** 1991. Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J Biol Chem **266:**2080-8.

157. **Shinabarger, D., T. B. May, A. Boyd, M. Ghosh, and A. M. Chakrabarty.** 1993. Nucleotide sequence and expression of the *Pseudomonas aeruginosa algF* gene controlling acetylation of alginate. Mol Microbiol **9:**1027-35.

158. **Simon, R., U. Priefer, and A. Puhler.** 1983. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. Nat Biotech **1:**784-791.

159. **Simpson, J. A., S. E. Smith, and R. T. Dean.** 1988. Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. J Gen Microbiol **134:**29-36.

160. **Simpson, J. A., S. E. Smith, and R. T. Dean.** 1989. Scavenging by alginate of free radicals released by macrophages. Free Radic Biol Med **6:**347-53.

161. **Slack, M. P., and W. W. Nichols.** 1981. The penetration of antibiotics through sodium alginate and through the exopolysaccharide of a mucoid strain of *Pseudomonas aeruginosa*. Lancet **2:**502-3.

162. **Soding, J., A. Biegert, and A. N. Lupas.** 2005. The HHpred interactive server for protein homology detection and structure prediction. Nucleic Acids Res **33:**W244-8.

163. **Song, Z., H. Wu, O. Ciofu, K. F. Kong, N. Hoiby, J. Rygaard, A. Kharazmi, and K. Mathee.** 2003. *Pseudomonas aeruginosa* alginate is refractory to Th1 immune response and impedes host immune clearance in a mouse model of acute lung infection. J Med Microbiol **52:**731-40.

164. **Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959-64.

165. **Struyve, M., J. Visser, H. Adriaanse, R. Benz, and J. Tommassen.** 1993. Topology of PhoE porin: the 'eyelet' region. Mol Microbiol **7:**131-40.

166. **Tamayo, R., J. T. Pratt, and A. Camilli.** 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu Rev Microbiol **61:**131-48.

167. **Tatnell, P. J., N. J. Russell, and P. Gacesa.** 1994. GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. Microbiology **140 (Pt 7):**1745-54.

168. **Thomas, S.** 2000. Alginate dressings in surgery and wound management--Part 1. J Wound Care **9:**56-60.

169. **Tian, J. Y., X. Q. Sun, and X. G. Chen.** 2008. Formation and oral administration of alginate microspheres loaded with pDNA coding for lymphocystis disease virus (LCDV) to Japanese flounder. Fish Shellfish Immunol **24:**592-9.

170. **Tikhonova, E. B., Q. Wang, and H. I. Zgurskaya.** 2002. Chimeric analysis of the multicomponent multidrug efflux transporters from gram-negative bacteria. J Bacteriol **184:**6499-507.

171. **Tremblay, J., A. P. Richardson, F. Lepine, and E. Deziel.** 2007. Self-produced extracellular stimuli modulate the *Pseudomonas aeruginosa* swarming motility behaviour. Environ Microbiol **9:**2622-30.

172. **Trujillo-Roldan, M. A., S. Moreno, D. Segura, E. Galindo, and G. Espin.** 2003. Alginate production by an *Azotobacter vinelandii* mutant unable to produce alginate lyase. Appl Microbiol Biotechnol **60:**733-7.

173. **Van Gelder, P., N. Saint, P. Phale, E. F. Eppens, A. Prilipov, R. van Boxtel, J. P. Rosenbusch, and J. Tommassen.** 1997. Voltage sensing in the PhoE and OmpF outer membrane porins of *Escherichia coli*: role of charged residues. J Mol Biol **269:**468-72.

174. **Van Gelder, P., N. Saint, R. van Boxtel, J. P. Rosenbusch, and J. Tommassen.** 1997. Pore functioning of outer membrane protein PhoE of *Escherichia coli*: mutagenesis of the constriction loop L3. Protein Eng **10:**699-706.

175. **Visudtiphole, V., D. A. Chalton, Q. Hong, and J. H. Lakey.** 2006. Determining OMP topology by computation, surface plasmon resonance and cysteine labelling: the test case of OMPG. Biochem Biophys Res Commun **351:**113-7.

176. **Wang, S. K., I. Sa'-Correia, A. Darzins, and A. M. Chakrabarty.** 1987. Characterization of the *Pseudomonas aeruginosa* alginate (*alg*) gene region II. J Gen Microbiol **133:**2303-14.

177. **Whitney, J. C., A. M. Neculai, D. E. Ohman, and P. L. Howell.** 2009. Expression, refolding, crystallization and preliminary X-ray analysis of *Pseudomonas aeruginosa* AlgE. Acta Crystallogr Sect F Struct Biol Cryst Commun **65:**463-6.

178. **Winsor, G. L., T. Van Rossum, R. Lo, B. Khaira, M. D. Whiteside, R. E. Hancock, and F. S. Brinkman.** 2009. Pseudomonas Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes. Nucleic Acids Res **37:**D483-8.

179. **Wong, T. Y., L. A. Preston, and N. L. Schiller.** 2000. Alginate Lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. Annu Rev Microbiol **54:**289-340.

180. **Wood, L. F., A. J. Leech, and D. E. Ohman.** 2006. Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: Roles of sigma (AlgT) and the AlgW and Prc proteases. Mol Microbiol **62:**412-26.

181. **Wood, L. F., and D. E. Ohman.** 2006. Independent regulation of MucD, an HtrA-like protease in *Pseudomonas aeruginosa*, and the role of its proteolytic motif in alginate gene regulation. J Bacteriol **188:**3134-7.

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

183. **Wood, S. R., A. M. Firoved, W. Ornatowski, T. Mai, V. Deretic, and G. S. Timmins.** 2007. Nitrosative stress inhibits production of the virulence factor alginate in mucoid *Pseudomonas aeruginosa*. Free Radic Res **41:**208-15.

184. **Worlitzsch, D., R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K. C. Meyer, P. Birrer, G. Bellon, J. Berger, T. Weiss, K. Botzenhart, J. R. Yankaskas, S. Randell, R. C. Boucher, and G. Doring.** 2002. Effects of reduced mucus oxygen concentration in

airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest **109:**317-25.

185. **Wozniak, D. J., T. J. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G. A. O'Toole, and M. R. Parsek.** 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 Pseudomonas aeruginosa biofilms. Proc Natl Acad Sci U S A **100:**7907-12.

186. **Xie, Z. D., C. D. Hershberger, S. Shankar, R. W. Ye, and A. M. Chakrabarty.** 1996. Sigma factor-anti-sigma factor interaction in alginate synthesis: inhibition of AlgT by MucA. J Bacteriol **178:**4990-6.

187. **Yamasaki, M., S. Moriwaki, O. Miyake, W. Hashimoto, K. Murata, and B. Mikami.** 2004. Structure and function of a hypothetical *Pseudomonas aeruginosa* protein PA1167 classified into family PL-7: a novel alginate lyase with a beta-sandwich fold. J Biol Chem **279:**31863-72.

188. **Ye, R. W., N. A. Zielinski, and A. M. Chakrabarty.** 1994. Purification and characterization of phosphomannomutase/phosphoglucomutase from *Pseudomonas aeruginosa* involved in biosynthesis of both alginate and lipopolysaccharide. J Bacteriol **176:**4851-7.

189. **Yorgey, P., L. G. Rahme, M. W. Tan, and F. M. Ausubel.** 2001. The roles of *mucD* and alginate in the virulence of *Pseudomonas aeruginosa* in plants, nematodes and mice. Mol Microbiol **41:**1063-76.

190. **Zhang, X., and H. Bremer.** 1995. Control of the Escherichia coli rrnB P1 promoter strength by ppGpp. J Biol Chem **270:**11181-9.

191. **Zielinski, N. A., A. M. Chakrabarty, and A. Berry.** 1991. Characterization and regulation of the *Pseudomonas aeruginosa algC* gene encoding phosphomannomutase. J Biol Chem **266:**9754-63.

192. **Zielinski, N. A., R. Maharaj, S. Roychoudhury, C. E. Danganan, W. Hendrickson, and A. M. Chakrabarty.** 1992. Alginate synthesis in *Pseudomonas aeruginosa*: environmental regulation of the *algC* promoter. J Bacteriol **174:**7680-8.

Appendix

MASSEY UNIVERSITY
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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Iain David Hay

Name/Title of Principal Supervisor: Prof. Bernd H. A. Rehm

Name of Published Paper: Bacterial biosynthesis of alginate.

In which Chapter is the Published Work: Chapter I

What percentage of the Published Work was contributed by the candidate: 90%

Candidate's Signature

27/9/11
Date

Principal Supervisor's signature

27/9/2011
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Name of Candidate: Iain David Hay

Name/Title of Principal Supervisor: Prof. Bernd H. A. Rehm

Name of Published Paper: Impact of alginate production on attachment and biofilm architecture of a supermucoid Pseudomonas aeruginosa strain.

In which Chapter is the Published Work: Chapter II

What percentage of the Published Work was contributed by the candidate: 40%

Candidate's Signature

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Name of Candidate: Iain David Hay

Name/Title of Principal Supervisor: Prof. Bernd H. A. Rehm

Name of Published Paper: Membrane topology of outer membrane protein AlgE, which is required for alginate production in Pseudomonas aeruginosa.

In which Chapter is the Published Work: Chapter III

What percentage of the Published Work was contributed by the candidate: 45%

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Name of Candidate: Iain David Hay

Name/Title of Principal Supervisor: Prof. Bernd H. A. Rehm

Name of Published Paper: MucR, a novel membrane associated regulator of alginate biosynthesis in Pseudomonas aeruginosa.

In which Chapter is the Published Work: Chapter IIII

What percentage of the Published Work was contributed by the candidate: 80%

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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Iain David Hay

Name/Title of Principal Supervisor: Prof. Bernd H. A. Rehm

Name of Published Paper: Identification of a periplasmic AlgK-AlgX-MucD multiprotein complex in Pseudomonas aeruginosa involved in biosynthesis and regulation of alginate

In which Chapter is the Published Work: Chapter V

What percentage of the Published Work was contributed by the candidate: 75%

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