Molecular Mechanism of Export of Alginate in *Pseudomonas aeruginosa*

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Read in the name of your lord who created man out of clot. Read and your lord is the most generous who taught by the pen. Taught man that which he knew not.

(Holy Quran)

Abstract

Pseudomonas aeruginosa is an opportunistic pathogen; infecting insects, plants and humans. It is of particular relevance to cystic fibrosis (CF) patients where it causes pulmonary infection and the leading cause of morbidity and mortality.

The CF lung environment selects for a variant of *P. aeruginosa* characterised by the overproduction of an exopolysaccharide called alginate. It has been hypothesized that outer membrane protein AlgE forms a channel through which alginate is secreted into the extracellular environment. Furthermore, studies have suggested that proteins involved in the polymerisation, modification and export of alginate form a multiprotein complex that span the bacterial envelope. The aim of this thesis was to investigate the role of AlgE in polymerisation and secretion of alginate. For this purpose *algE* knockout mutant was created in PDO300. Results showed that AlgE does not have a role in alginate polymerisation however it has a role in secretion of alginate and stability of the alginate biosynthesis machinery. By performing FLAG epitope insertion mutagenesis the topology of AlgE was verified and site-directed mutagenesis further showed that the positive electrostatic field inside the AlgE lumen is required for efficient secretion of negatively charged alginate. By employing mutual stability analysis, evidence was provided for the existence of trans-envelope multiprotein complex required for alginate biosynthesis. Co-immuniprecipitaion assay suggest that AlgE interacts with periplasmic located AlgK and, most probably, this interaction is mediated by the peripasmic turn 4 of AlgE. Pull-down assays further showed that AlgK interacts with another periplasmic protein AlgX which in turn interacts with the inner membrane protein Alg44. Based on mutual stability analysis it was proposed that Alg44 interacts with Alg8 which might interacts with AlgG as well. Our results also support the existence of internal promoters for AlgE and AlgG.

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 7, published by the Massy University doctoral research committee (January 2011).
The following sections of this thesis have been published in internationally peer-reviewed journals.

Chapter II

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

Chapter III

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Chapter IV

Rehman ZU, Rehm BHA (2013). The dual roles of *Pseudomonas aeruginosa* AlgE in secretion of the virulence factor, alginate, and formation of the secretion complex Applied and Enviornmental Microbiology **79:** 2002-2011.

Chapter V

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Contributions Rehman ZU made to publications are as follows

Chapter II: This review was drafted by I.D.H, Z.U.R and A.G and finalised by B.H.A.R

Chapter III: Plasmid pEX100T:Δ*algE* was made by U.R. Plasmid pBBR1MCS5:*algE* was made by IDH. PDO300Δ*algE* was made by Z.U.R and complementation was done by Z.U.R. FLAG tag variants of *algE* were made by Z.U.R. Alginate quantification was done by Z.U.R. Outer membrane protein isolation and immunoblotting was done by I.D.H. Manuscript was drafted by I.D.H and Z.U.R and finalised B.H.A.R.

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DNA sequencing was provided by external services.

This is to certify that above mentioned work was conducted by Zahid Ur Rehman.

Signature Date Signature Date

Prof. Bernd H.A. Rehm Zahid ur Rehman

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Chapter I

General introduction to polysaccharides

Polysaccharides are one of the most abundant and important classes of biopolymers produced by bacteria. They are composed of monosaccharides or the repeat units linked to each other through glycosidic bonds to form long chains. Polysaccharides confer survival advantages to the producing organism under certain environmental conditions and contribute towards pathogenicity. Polysaccharides are water soluble, biocompatible and biodegradable and therefore are becoming increasingly important in various biomedical applications such as in dentistry, ophthalmology, tissue engineering, organ transplant and controlled release of drugs. Traditionally, polysaccharides are harvested from plant and marine algae and are used as viscosifying, stabilising and gelling agents in a range of food and cosmetic products (160). Medical applications of polysaccharides require consistent material properties that can be hard to obtain from plants and marine sources. However, bacterial systems can be genetically engineered with relative ease to produce tailor-made polysaccharides with the required material properties. Complex regulatory mechanisms exist to control the biosynthesis of these polymers (130). Understanding the mechanism of regulation and biosynthesis of various polysaccharides will not only provide insight into bacterial pathogenesis but also contribute to the design of commercially and medically important polysaccharides (130).

Polysaccharides can be divided into groups based on their cellular location or repeat unit composition. Alginate and cellulose are examples of exopolysaccharides that are secreted into the growth medium. Capsular polysaccharides, such as K-antigens, remain tightly associated with the cell surface whereas intracellular polysaccharides, such as glycogen, reside in the cytosol. On the basis of repeat unit composition polysaccharides are divided into: repeat unit polymers (xanthan and pel); repeating polymers (cellulose) and non repeating polymer (alginate) (130).

Although polysaccharides play an important role in the life of bacteria, the mechanisms involved in their biosynthesis and secretion are only now beginning to be understood. Emerging evidence suggests that the biosynthesis and translocation of polysaccharides to the cell surface are temporally and spatially coupled by multiprotein complexes spanning the cell envelope. This reduces the problems associated with the translocation of newly synthesised polysaccharide across the bacterial envelope into the surrounding enviornment. Most of our current knowledge of the assembly of bacterial polysaccharide is based on studies conducted on the capsular K-antigen of *Escherichia coli* and the lipopolysaccharide (LPS) O-antigen of *Salmonella enterica* (183). It is

therefore not surprising that assembly of these polysaccharides serve as a model for studying the biosynthesis and translocation of other polysaccharides. However, significant differences exist in the mechanism by which various polysaccharides are synthesised and translocated across the envelope (130).

Biosynthesis of extracellular polysaccharides

Based on current knowledge, mechanisms for biosynthesis of polysaccharides are divided into three general pathways described as the "Wzx/Wzy dependent pathway", "ATP-binding cassette (ABC) transporter dependent pathway" and the "synthase dependent pathway". The best studied of which is the Wzx/Wzy dependent pathway that is used in the assembly of polysaccharides, such as, LPS O-antigen and group 1 and 4 capsular polysaccharides (CPS) (32). In this pathway, polysaccharide repeat units are first assembled onto an undecaprenyl phosphate (Und-P) lipid carrier. This reaction takes place at the cytoplasmic face of the membrane where activated sugar-nucleotide precursors are used by membrane-embedded glycosyltransferases to build the repeat unit. The Und-PP linked repeat unit is transported across the inner membrane by the activity of the Wzx protein "flippase"(98, 183). On the periplasmic side of the inner membrane, the lipid-linked repeat units are added to the reducing end of the growing polysaccharide chain. This polymerisation reaction is catalysed by a membrane-embedded putative polymerase Wzy (193). For the biosynthesis of the LPS O-antigen the polymerisation activity is influenced by another protein referred to as polysaccharide co-polymerase (PCP) which helps regulate the chain length of the O-antigen (45). In the biosynthesis of the CPS K-antigen, co-polymerase Wzc interacts with Wza, which belongs to family of the proteins termed as outer membrane polysaccharide export proteins (OMPX), to form a continuous channel for the secretion of polysaccharide (Fig. 1) (27, 115).

The ABC transporter dependent pathway is involved in the biosynthesis and export of *E. coli* group 2 and 3 capsular polysaccharide K-antigens (32). The ABC transporters involved in the translocation of polysaccharides are integral membrane proteins that are located in the inner membrane with the nucleotide binding domains residing in the cytoplasm. For this system, the entire polysaccharide is assembled on the lipid carrier at the cytoplasmic face. The type of the lipid carrier depends upon the polysaccharide

being synthesized (32). For CPS K-antigen synthesized by ABC transporters, diacylglycerol phosphate is used as a carrier for the translocation (183). The polysaccharide is transferred across the cytoplasmic membrane by utilising the energy of ATP hydrolysis. Despite these differences from the Wzx/Wzy dependent system, similar proteins are shared between these pathways for the translocation of polysaccharide through the periplasm and outer membrane. Similar to the Wzx/Wzy dependent pathway, a co-polymerase (PCP) has been suggested to interact with OMPX forming a continuous channel for the export of newly synthesised polymer (Fig. 1) (33, 113).

The synthase dependent pathway is the least understood mechanism for the assembly and export of polysaccharides. However, this pathway is used in the assembly of many commercially and medically important polysaccharides such as cellulose, hyaluronic acid and alginate. The polymerisation activity is dependent on a family 2 glycosyltransferase, usually located in the inner membrane. Synthesis of polysaccharide may or may not require a lipid acceptor (20, 72). Until now, it remained unclear how the polysaccharide is transported across the inner membrane after polymerisation. Recently, by employing an *in vitro* assay, reconstituting hyaluronan synthase into proteoliposomes, it was shown that synthase alone is sufficient for the elongation and translocation of hyaluronic acid across the membrane (78). In the biosynthesis of alginate, the polymerisation activity of "synthase", Alg8, is influenced by a c-di-GMP binding protein Alg44, a putative co-polymerase. Although Alg44 has been proposed to be required for the translocation of alginate across the periplasm its role in the translocation across the inner membrane is unclear (72). In the biosynthesis of cellulose, a multifunctional protein BcsA harbouring both a glycosyltransferase domain and a c-di-GMP binding domain is thought to facilitate both polymerisation and translocation across the inner membrane. In this sense, BcsA can be considered to represent a fusion of Alg8 and Alg44 (145, 185). Once in the periplasm, a tetratricopeptide-repeat containing protein guides the polysaccharide towards an outer membrane (OM) β-barrel porin for secretion from the cell (Fig. 1) (69, 188).

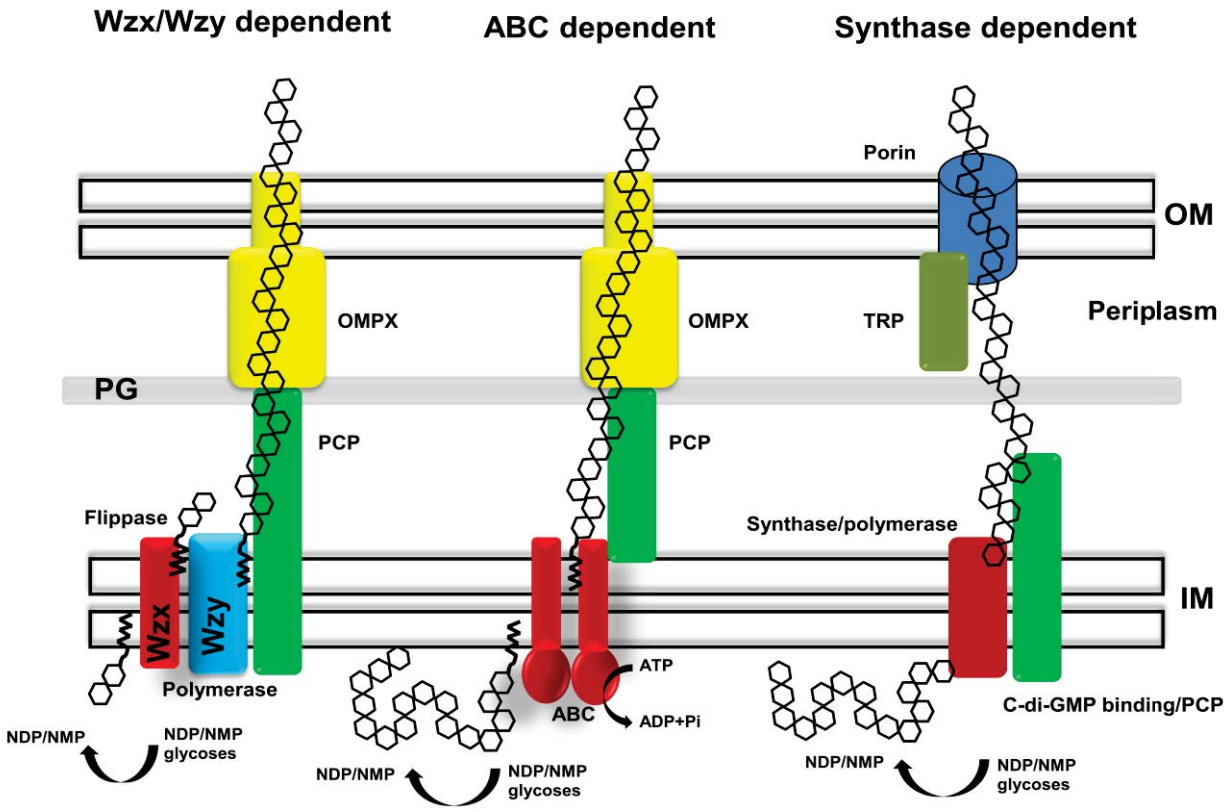

Fig. 1: Schematic representation of polysaccharide biosynthesis and secretion pathways.

In the Wzx/Wzy dependent pathway, glycosyltransferases build a repeat unit onto a lipid carrier which is flipped across the inner membrane by Wzx. The repeat unit is transferred to the growing polysaccharide chain by the activity of Wzy. Polysaccharide is secreted from the cell utilising the scaffold formed by the family members of the PCP and OMPX families. For ABC dependent pathway, the complete polysaccharide is built on a lipid carrier at the cytoplasmic side of inner membrane before being transferred across the membrane. The polysaccharide is exported out of the cells using a translocation pathway formed by PCP and OMPX family members. In the synthase dependent pathway, usually a synthase is capable of both polymerisation and translocation of the newly formed polymer across the inner membrane. A c-di-GMP binding protein regulates the activity of the synthase. A TRP containing and OM β-barrel porin is usually involved in the export of polysaccharide across the periplasm and OM (181). Abbreviations: PCP, Polysaccharide co-polymerase; OMPX, outer membrane polysaccharide export; TPR, tetratricopeptide-repeat containing protein; NDP/NMP, nucleotide di-phosphate/nucleotide mono-phosphate; OM, outer membrane; IM, inner membrane; PG, peptidoglycan.

Polysaccharides produced by *Pseudomonas aeruginosa*

P. aeruginosa is a versatile organism that thrives in a wide range of environments. It is an opportunistic pathogen of plants, animals and humans and can cause acute and chronic infections. It is especially important in cystic fibrosis (CF) patients where it establishes chronic infections and forms biofilms. Biofilms are surface-attached microbial communities resistant to most antibiotics and the host immune defence system. To date three important exopolysaccharides have been identified in *P. aeruginosa* namely Pel, Psl and alginate. Interestingly the forced production of Psl reduces the amount of alginate produced and induction of Pel leads to a reduction in the amount of Psl produced (102). It appears that amount of each polysaccharides is not regulated at the transcriptional level but at the sugar-nucleotide precursor level (102). These polysaccharides play a critical role in the life of the bacterium, mainly serving as matrix components during biofilm formation. *P. aeruginosa* isolated from CF patients can display a mucoid colony phenotype which is caused by the overproduction of an extracellular polysaccharide called alginate (48). However, some CF isolates exhibit a rugose small colony variant phenotype (RSCV) due to the production of Pel and Psl polysaccharides. Most non-CF isolates of *P. aeruginosa* produce Pel and Psl polysaccharide. The biosynthetic operons for these two polysaccharides were originally identified as a result of whole genome sequencing of *P. aeruginosa* (168). The name Psl stands for **p**olysaccharide **s**ynthesis **l**ocus while Pel derives its name due to its involvement in the formation of microbial mats (pellicles) at the air-liquid interface (48).

CF lungs are initially infected by non-mucoid *P. aeruginosa* which over the course of infection switch to a mucoid phenotype, which is related to alginate production. Although alginate does not seem to be required for formation of biofilm, its presence has an impact on the architecture of biofilms (194). Alginate produced by *P. aeruginosa* is acetylated, which is required for alginate to exert its role in the formation of structured biofilms (118). Although alginate is an important component of a mature biofilm, overproduction of alginate impairs the initial attachment of cells to abiotic surfaces (68). The biofilms formed by mucoid *P. aeruginosa* are more resistant to antimicrobial agents as well as to interferon-gamma mediated phagocytosis, by the cells of immune system (96). Using *Caenorhabditis elegans* as a model organism it was shown that alginate helps to mask the producing cell from recognition by NPR-1

neuropeptide receptors. It was suggested that a similar masking strategy may be used by *P. aeruginosa* to evade the host immune response (127). It has been observed that biofilms formed by alginate deficient cells contain a high proportion of dead cells suggesting that alginate has a role in the viability of cells (54).

Although our knowledge about the biosynthesis and regulation of Pel and Psl is scant, recent studies have highlighted their importance in the formation of biofilms (52, 101). It has been suggested that during biofilm formation, Pel polysaccharide plays a structural role in maintaining cell to cell interactions and a protective role by conferring resistance to aminoglycoside antibiotics (28). Psl plays an important role in initial attachment to biotic and abiotic surfaces and prevents efficient opsonisation and phagocytic killing of *P. aeruginosa* (101, 109). Although it appears that Pel and Psl have distinct role in biofilm formation, these polysaccharides can perform structurally redundant functions in mature biofilms (29). Due to the recent discovery of the Pel and Psl loci and their important roles in biofilm formation, studies have only just begun to unravel their mechanism of biosynthesis and very recently models for the biosynthesis of Pel and Psl have been proposed. These models are based on the predicted structures of proteins involved in Pel and Psl biosynthesis and their homology to the proteins of well characterised polysaccharides biosynthesis pathways (48).

Pel biosynthesis

The exact structure and composition of Pel polysaccharide is not known but it has been proposed to be rich in glucose. The genes involved in the biosynthesis and translocation of Pel polysaccharide constitute an operon, *PelA*/*B*/*C*/*D*/*E*/*F*/*G*, which consists of seven genes (52). Surprisingly there appears to be no gene in this operon involved in the generation of a sugar-nucleotide precursor. However, structural homology modelling has identified a cytoplasmically located putative glycosyltransferase, PelF, involved in the polymerisation of polysaccharide (31). The cytoplasmic membrane protein PelD, carrying a c-di-GMP binding PilZ domain, is involved in the post-translational regulation of polysaccharide production (95). Two other proteins, PelG and PelE, have been predicted to be inner membrane proteins involved in modification and translocation of the polymer. A predicted periplasmic and/or OM protein, PelB, carries an extensive tetratricopeptide-repeat structure and may serve as a scaffold for the

assembly of the Pel biosynthesis and secretion complex (48). PelC is an outer membrane protein and its C-terminal alpha-helix is required for insertion into the OM in a manner similar to Wza of capsular polysaccharide biosynthesis (180). However, structural modelling of the C-terminus of PelC has suggested that it interacts with the peptidoglycan in a fashion similar to TolB of *E. coli* (12). Finally, PelA has been predicted to contain a glycoside hydrolase domain and a carbohydrate esterase domain which are involved in the modification of polymer after synthesis (177). A model has been proposed for the assembly of the Pel polysaccharide biosynthesis and secretion complex. This models suggest that biosynthesis of Pel is more closely related to alginate biosynthesis, with notable differences, than to Wzx/Wzy dependent group 1 capsular polysaccharide biosynthesis pathway This model predicts the localization and function of protein in the cell as shown in fig. 2.

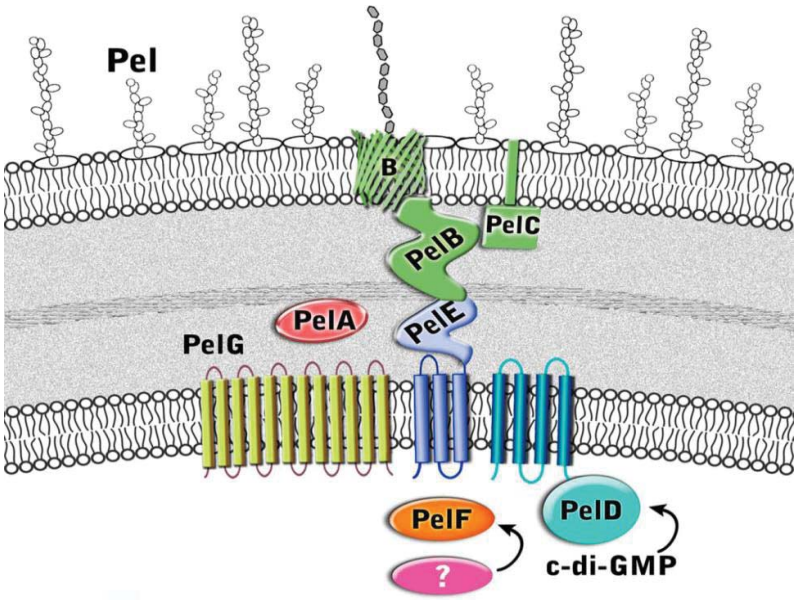

Fig. 2: Proposed model for Pel biosynthesis. (Figure adapted from Franklin, M. J *et al* 2011)(48).

Psl biosynthesis

Psl polysaccharide is composed of D-mannose, L-rhamnose and D-glucose. The Psl biosynthesis locus is composed of 12 genes namely *PslA*/*B*/*C*/*D*/*E*/*F*/*G*/*H*/*I*/*J*/*K*/*L*. PslB is a dual-functional enzyme which possess both phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activities. With the exception of PslB, deletion of any of the genes of the Psl biosynthesis operon resulted in loss of Psl production which suggests that PslB function can be complemented by other cellular proteins (19). As a double mutant of *pslB* and *wbpW* (having both PMI and GMP activities) results in the loss of Psl production, this indicates that the function of PslB and WbpW is interchangeable. PslB is the only protein encoded by this operon which is involved in the sugar precursor biosynthesis (GDP-mannuronic acid), so it is possible that proteins including AlgC, RmlC and GalU can be used for the generation of remaining activated sugar-nucleotides (Fig. 2) (19). The proteins PslF, PslH, PslI and PslC have been predicted to contain glycosyltransferase domains and are involved in the formation of repeat units for Psl polysaccharide. PslA is thought to have polyprenyl glycosylphosphotransferase activity and provide the site for the assembly of the oligosaccharide repeat unit onto the isoprenoid lipid. PslE has been predicted to be a polysaccharide co-polymerase which controls the polysaccharide chain length. It has been hypothesized that PslJ has a function similar to the Wzy glycosyltransferase and PslK acts as a flippase, which transfers the repeat unit across the inner membrane into the periplasm. Sequence homology searches have suggested that PslL has an acyltransferase domain. However, the function of these proteins remains to be confirmed by experimentation. Assigning function to PslD remains difficult but it has been suggested to form a pore, with the help of PslE, in the OM through which polysaccharide is secreted into the extracellular environment (48). On the basis of homology and structural modelling a model has been proposed for the biosynthetic mechanism of Psl polysaccharide production. According to this model, proteins involved in Psl biosynthesis bear structural similarities to the biosynthetic proteins of *E. coli* group 1 capsular polysaccharides suggesting Psl biosynthesis is closely related to the Wzx/Wzy dependent mechanism (48). This model predicts the localization and role of proteins involved in Psl biosynthesis (Fig. 3).

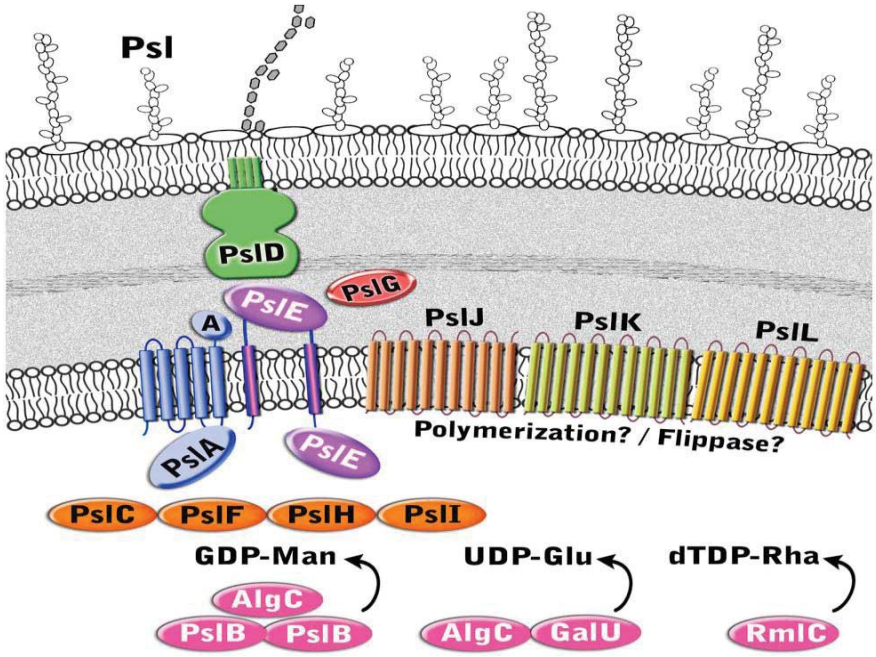

Fig. 3: Proposed model for Psl biosynthesis. (Figure adapted from Franklin, M. J *et al* 2011)(48).

Alginate biosynthesis

Alginates mechanism of biosynthesis and regulation is one of the best characterised of the polysaccharides produced by *P. aeruginosa*. Alginate produced by *P. aeruginosa* was first identified in 1964 from clinical isolates from CF patients (97). In CF patients, alginate not only confers resistance against antibiotics and host immune defences but also enters narrow airways and can cause death due to suffocation. Because of the importance of alginate in CF patients, the mechanism of biosynthesis and regulation has been actively studied by researchers in a hope to find suitable targets for drug and vaccine development. Originally, it was proposed that proteins involved in the biosynthesis of alginate were encoded by an operon consisting of twelve genes (*algD*/*8*/*44*/*K*/*E*/*G*/*X*/*L*/*I*/*J*/*F*/*A*) under the control of the *algD* upstream promoter (48). However, recently the possibility of internal promoters for *algE*, *algI* and *algG* has also been suggested (26, 122). Another gene, *algC*, involved in alginate biosynthesis is located elsewhere on the genome. A recent study has shown that AlgC plays an important role in regulating the amount of alginate, Pel, Psl and lipopolysaccharide,

possibly by controlling sugar-nucleotide precursor levels (102). The current model and details of alginate biosynthesis, its regulation and potential commercial and medical applications are discussed in detail in the subsequent chapters.

Outer membrane proteins

Outer membrane proteins (OMPs) are synthesized in the cytoplasm with an N-terminal signal sequence which directs them to the cytoplasmic membrane for secretion through energy-dependent Sec translocons. After cleavage of the N-terminal signal sequence in the periplasm OMPs are sequestered either by SurA or Skp-Degp which transport them to the Beta-barrel Assembly Machinery (BAM complex) located in the OM (89). The BAM complex recognises its substrate through interaction with the C-terminal amphipathic β-strand of an unfolded protein (142). In *E. coli* the BAM complex consists of five proteins BamA, BamB, BamC, BamD and BamE which are involved in the folding and insertion of β-barrel proteins in the OM (182). In *P. aeruginosa* two members of this BAM complex have been identified to date, BamA (Opr86) and BamE (OmlA) (119, 171). The exact mechanism involved in folding and insertion of proteins into the OM has not yet been determined. However research has shown that folding and insertion is energy independent.

Most β-barrel proteins composed of only single polypeptide chain have large extracellular loops and short periplasmic turns. Most of the amino acid residues located in the extracellular loops and periplasmic turns are charged or hydrophilic and are solvent exposed. However the β-strands, which traverse the membrane, are composed of alternative hydrophobic and hydrophilic amino acids. The hydrophobic residues face into the hydrophobic lipid bilayer, whilst charged residues face the inside of the barrel (141).

Outer membrane proteins make almost 50% of the mass of outer membrane. The OMPs are either integral membrane proteins or lipo-proteins attached to the OM via an N-terminal lipid moiety. Some of the OMPs, called porins, form pores for the transport of various substances across the OM (90). Porins can be either general porins such as OprF or substrate specific porins such as OccD and OccK family members in *P. aeruginosa* (62, 99). General porins do not show any specificity towards its substrate while substrate specific proteins are selective and allow the transport of a narrow range of

substartes. Apart from porins some proteins located in the OM are involved in the uptake of iron-sidrephore complex in an energy dependent mechanism and are called TonB dependent receptors, for instance, FpvA of *P. aeruginosa* (59). Furthermore, some proteins located in the OM form secretion channels e.g autotransportes, MexAB-OprM channel for the efflux of antibiotics and type I to VI secretion systems for the extracellular release of proteins and virulence factors (62, 90).

General and substrate specific porins are composed of single peptide chain which forms a channel in the OM with large extracellular loops and short periplasmic turns. General porins are usually composed of 16 strands of antiparallel β-sheets and although these pores are non-specific they can show some selectivity towards anions or cations (90). Transport of substrate through general porins occurs across the concentration gradient i.e from higher solute concentration to lower solute concentration. Substrate specific porins e.g LamB of *E.coli* consist of of 18-stranded anti-parallel β-barrel and form homotrimers in the OM (152). Substrate specific porins usually have binding sites for their substrate inside the barrel lumen which allow the transport of substrates against the concentration gradient (90). In most of the general and substrate specific porins the extracellular loop 3 is bent inside the barrel lumen to form a constriction channel which limits the size of substrate that can be transported (90).

An exception to OM β-barrel conformation is, Wza which is required for the translocation of group 1 capsular polysaccharide. X-ray crystal structure analysis has shown that Wza forms a octameric α-hellical barrel in the OM (38). AlgE is also an OM protein which has been proposed to form a 18-stranded β-barrel pore and has a role in the secretion of alginate. In this thesis, in particular, we have investigated the following questions. What is the specific function of AlgE in secretion of alginate? Does AlgE forms a β-barrel pore in the outer membrane? What is the role of extracellular loops in the stability of AlgE and its substrate specificity? Do the positive charge residues play a role in the efficient scretioin of negatively charged alginate? Does AlgE interact with other components of proposed periplasmic scaffold required for secretion of alginate? What periplasmic turns of AlgE might have a role in mediating interaction of AlgE with other component? Apart form these questions we have provided strong evidence for the existence of trans-envelope multiprotein complex involved in polymerisation, modification and export of alginate.

Chapter II

Bacterial biosynthesis of alginates
(Review of literature)

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Abstract

Alginates are polysaccharides with many industrial and medical uses, from food additives to the encapsulation agents in 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. Here we discuss 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 lack the desired material properties (92, 112).
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 (68, 118). 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 (123, 164-167). 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 (64).

As alginates become used in increasingly more applications, both in medical and industrial fields it is becoming desirable to better control the material properties of these alginates. Due to a better understanding of the polymerisation, modification, and regulation of alginate biosynthesis in these bacteria, and the relative ease of optimising

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 been used to modify the G content and thus material properties of seaweed derived alginates (112), and alginates from mutant *Pseudomonas fluorescence* can be produced that lack any G residues (polymanuronan) (22, 56).

Genetics of bacterial alginate biosynthesis

The work of Darzins and Chakrabarty (35) first demonstrated many of 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* (128) (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 (57). 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* (135, 155, 159). 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 (51), *algG* for epimerization (46), and *algL* for degradation (151)); the putative outer membrane porin (*algE*) (131, 133). The products of the *alg8* gene is 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 polymerisation process (140). The product of the *alg44* gene is thought to be involved in the post-translational regulation of alginate (70, 138). 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 (61, 81). 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 (57, 121, 197) and expression is driven from its own promoter (199). 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) (128).

Gene	Gene product	Reference
algD	GDP mannose dehydrogenase	(172)
alg8	Glycosyltransferase/polymerase function?	(137)
alg44	c-di-GMP binding regulation/ membrane fusion?	(138)
algK	Periplasmic scaffold?	(81)
algE (algJ)	Outer membrane alginate porin?	(129, 131)
algG	Mannuronan C-5-epimerase	(46)
algE1-E7	*Azotobacter* extracellular epimerases	(42)
algX	Periplasmic unknown function/scaffold/sequesters MucD	(61)
algL	Alginate lyase	(82)
algI	O-Acetylation	(51)
algJ (algV)	O-Acetylation	(51)
algF	O-Acetylation	(51)
Alga	Phosphomannose isomerase/GDP mannose pyrophosphorylase	(161)
algB	Member of ntrC subclass of two-component regulators	(103)
algC	Phosphomannomutase	(197)
algH	Unknown	(9)
algR	Regulatory component of two-component sensory transduction system	(103)
algQ	Histone like transcription regulator. AKA *algR2*	(84)

algP	Histone-like transcription regulator. AKA *algR3*	(85)
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}	(195)
mucA	Anti σ factor	(195)
mucB	Anti σ factor	(21)
mucC	Regulator?	(15)
mucD	Homologue of *E. coli* serine protease DegP	(191)
algW	Homologue of *E. coli* serine protease DegS	(21)
mucP	Homologue of *E. coli* RseP protease involved in AlgU RIP cascade	(125)
mucE	Periplasmic or outer membrane protein involved in AlgU RIP cascade	(125)
mucR	Alginate specific diguanylate cyclase (c-di-GMP synthesizing)	(70)

Biosynthesis of alginate

The first bacterial alginate biosynthesis pathway was proposed in 1975 by Pindar and Bucke in *A. vinelandii* (124). 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 (139) (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 6 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 (100, 114). 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) (107). AlgC (phosphomannomutase) directly converts mannose-6-phosphate to mannose-1-phosphate (198). The GDP-mannose pyrophosphorylase (GMP) activity of AlgA (PMI-GMP) catalyses the conversion of the activated mannose-1-phosphate to GDP-mannose with the hydrolysis of GTP (161). 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 polymerisation, GDP-mannuronic acid (144). 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 (172).

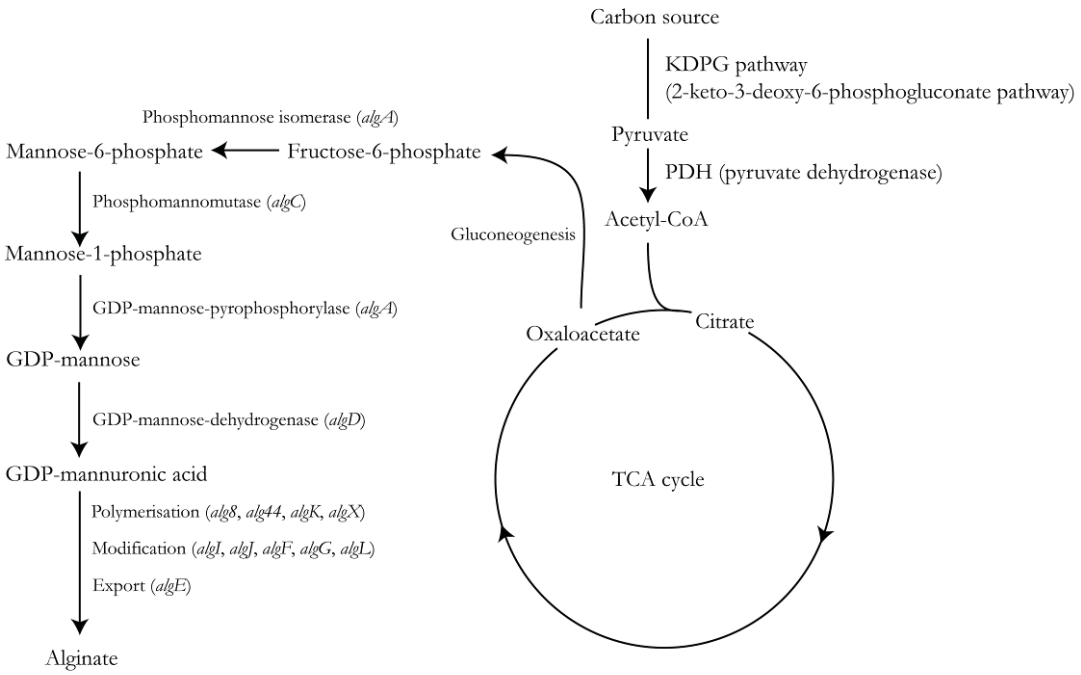

Fig. 1: Proposed alginate biosynthesis pathway in *P. aeruginosa.* Figure adapted from Remminghorst & Rehm, 2006 (139).

Polymerisation 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* (140). 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 (140, 150). Furthermore, structural similarities were observed when compared to functionally related enzymes such as AcsAB (cellulose synthase) (149) and Chs1 (chitin synthase) (139). 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 multiprotein 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, 56, 69, 80, 143). 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 polymerisation process but may be playing more of a structural/protection from alginate lyase degradation (discussed below) role (56, 82, 143).

It has been suggested that Alg8 and Alg44 interact and play an important role in the polymerase complex (147). Recently, it was shown in complementation studies that Alg44 is required for alginate polymerisation. 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 (138) (Fig. 2). Additionally the cytosolic N-terminal half of Alg44 has been shown to possess a functional and essential PilZ domain (108, 138). PilZ domains are involved in binding of the regulatory molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (4). 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 suggested that alginate is synthesised as polymannuronate and that modification occurs at polymer level. Three classes of alginate modifying enzymes have been described: transacetylases (47, 49, 51, 162), mannuronan C-5-epimerases, (22, 46, 56) and lyases (3, 6). In *P. aeruginosa* AlgI, AlgJ (called AlgV in *Azotobacter*), and AlgF were shown to form the acetylation complex (47, 49, 51, 162). Transacetylation occurs at the O2' and/or O3' 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 (46, 49, 189). Thus, the acetylation of alginate indirectly controls epimerisation and length of the alginate polymer.

Mannuronan C-5-epimerase introduces guluronic acid residues in to 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 (42, 132). 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 (42, 76, 146).

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 (3) 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 (82) 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* (176). It has been suggested that AlgL may function in controlling the length and molecular weight of the alginate polymer (17), providing short alginate polymers to "prime" the polymerisation of alginate (17), or to clear the periplasm of misguided alginate (6, 82). 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* (189)). These enzymes have differing residue specificities and cellular localisations. Among *Pseudomonas* species there has been one other protein identified to have alginate lyase activity, PA1167 (196). Among *Azotobacter* species at least four other lyases have been identified: the epimerase AlgE7, and three others, AlyA1, AlyA2 and AlyA3 (55).

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 (131). 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 (58, 69).

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, contain 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 (23, 37, 44, 126). This region shows high similarity to the well characterised 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 (190). 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 . 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 of 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 (94, 106, 155, 157). 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 (21, 192). 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 (104, 106), 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 (21, 192) 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 the the C-terminus of particular misfolded proteins, in particular a periplasmic or outer membrane located protein called MucE (125). A third periplasmic protease, MucD, appears to be playing a role antagonistic to that of AlgW. Deletion mutations of MucD lead to a mucoid phenotype indicating a negative regulatory role (191). MucD appears to be involved in the degradation of misfolded proteins in the periplasm that would outherwise activate (de-repress) AlgW (125, 192) (Fig. 2). The role of MucC remains unclear. It should be noted that the alternate sigma factor, AlgU, released in this RIP cascade does not exclusively activate transcription of the alginate operon but is involved in the the transcription of other genes with diverse functions (43, 44).

There appears to be an additional 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 (16). 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 frameshift 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 (103). AlgB is also part of a two component regulator and binds to one site on the *algD* promoter, again its activity is apparently independent of its sensor kinase KinB (103). 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 (108, 138). 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* (70) (Fig. 2).

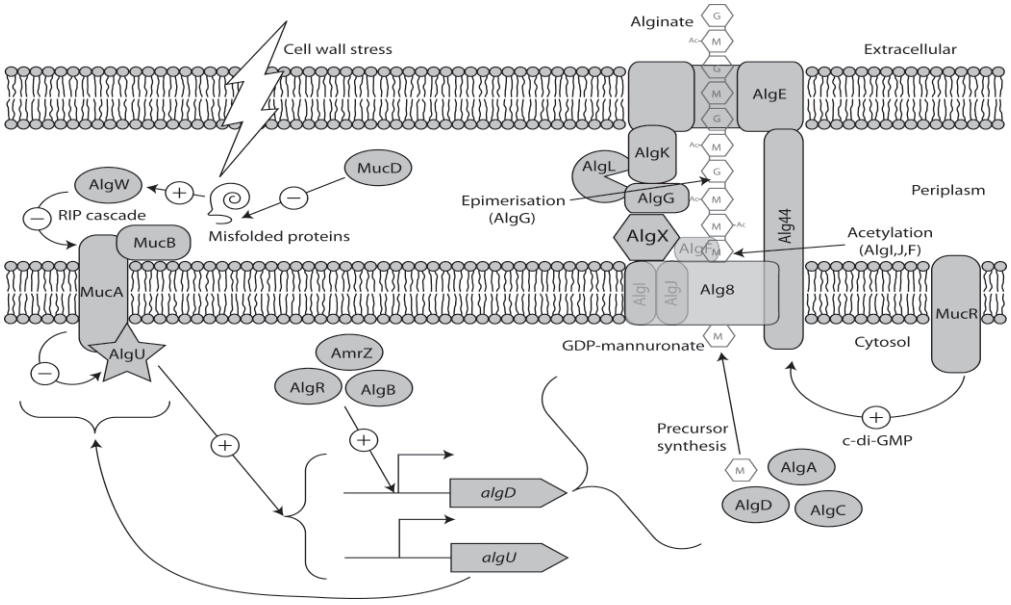

Fig. 2: Overview of alginate biosynthesis in *P. aeruginosa*.

Applications of bacterial alginates

Since its discovery alginate has been used for many commercial purposes. Due to its water holding, gelling, viscosifying and stabilising properties, alginate of algal origin is used extensively in the food industry as a food additive. Based on its high biocompatibility (88), abundance and availability of the source, and relatively low cost to produce, alginates are increasingly being used in the medical field (173). 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 re-apply, these dressings are advantageous as they do not adhere to the wound and can be removed with a saline wash (173). Alginate has also been used for the controlled release of drugs; the drugs are entrapped in alginate beads, from which the drug can by slowly released (136). 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 (64-67, 87). Also, recently alginate encapsulation has been used successfully as a method to orally deliver DNA based vaccines (174). To date the vast majority of the alginate used for commercial and medical purposes is 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 which require extensive downstream processing to remove. The relative 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 (135, 147). These alginates could be directly produced from the bacteria or produced from the cheaper seaweed source or as polymannuron from *P. fluorescens* (22, 56) and later modified with bacterial enzymes. Indeed this has already been demonstrated with the use of the extracellular epimerases secreted by *Azotobacter vinelandii* to treat polymannuron to produce alginates not found in nature with useful material properties (111, 112).

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 virulence factor during chronic infection of Cystic Fibrosis patients. Here it was demonstrated that the outer membrane protein AlgE is required for 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 is occurring but 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 an ubiquitous opportunistic human pathogen responsible chronic infections of the lungs of Cystic Fibrosis (CF) patients, where it is the leading cause of mortality and morbidity (30). The establishment of a chronic infection in the lungs of CF patients coincides with *P. aeruginosa* switching to a stable mucoid variant, producing copious amounts of the exopolysaccharide alginate; this is typically a poor prognosis indication for these patients (105, 133). Alginate is a linear unbranched exopolysaccharide consisting of 1,4-linked monomers of β-D-mannuronic acid and its C5-epimer α-L-guluronic acid, which is known to be produced by only two bacterial genera, *Pseudomonas* and *Azotobacter* (139). The switch to a mucoid phenotype coincides with the appearance of 54 kDa protein in the outer membrane; this protein has been identified and designated as AlgE (58, 133).

The genes encoding the alginate biosynthesis machinery are located within a 12 gene operon (*algD-8-44-K-E-G-X-L-I-J-F-A*). AlgA and AlgD, along with AlgC (not encoded in the operon), are involved in precursor synthesis (139). Alg8 is the catalytic subunit of the alginate polymerase located at the inner membrane (140). AlgG is a C-5-mannuronan epimerase (80). AlgK contains four putative Sel1-like repeats, similar to the tetratricopeptide-repeat motif often found in adaptor proteins involved in the assembly of multiprotein complexes (10, 34). AlgX shows little homology to any known proteins and its role is unclear (60). Knock-out mutants of AlgK, AlgG or AlgX have non-mucoid phenotypes, although they produce short alginate fragments, due to the activity of the alginate lyase (AlgL), which degrades the nascent alginate (3, 60, 80-82, 143). AlgF, AlgI and AlgJ are involved in acetylation of alginate, but are not ultimately required for its production (51). 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 (70, 108, 138). The periplasmic C-terminus of Alg44 shares homology with membrane fusion proteins involved in bridging the periplasm in multi-drug efflux pumps (40, 175). The periplasmic alginate lyase, AlgL, appears to be required for translocation of intact alginate across the periplasm (3, 110). AlgE is an outer membrane, anion selective channel protein through which alginate is presumably secreted (131). A protein complex or scaffold through which the alginate chain can pass and be modified which spans the periplasm bridging

the inner membrane located (Alg8) to the outer membrane pore (AlgE), has been proposed (82). Indeed, it has been demonstrated that both the inner and outer membrane are required for the *in vitro* polymerization of alginate (140).

The requirement of the AlgE for the biosynthesis of alginate in *P. aeruginosa* was first observed by complementation of an alginate negative mutant, derived from chemical mutagenesis, with a DNA fragment containing *algE* (26). Secondary structure predictions suggested that AlgE forms an 18 stranded β-barrel with extended extracellular loops. Several of these loops show a high density of charged amino acids suggesting a functional role in the translocation of the anionic alginate polymer (129, 131). Preliminary steps towards the crystal structure of AlgE have been reported (186).

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. *Escherichia coli* strains were grown in LB medium at 37°C. *E. coli* S17-1 was used for conjugative transfer of the suicide plasmid pEX100T:Δ*algE*ΩGm and the flipase-encoding plasmid pFLP2. When 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 (PIA) 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 liter) at 37°C and, where required, antibiotics were added to appropriate concentrations. The antibiotic concentrations used for *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.).

Isolation, analysis, and manipulation of DNA.

General cloning procedures were performed as described previously (148). All pBBR1MCS-5 derived plasmids were transferred to *P. aeruginosa* strains via electroporation as previously described (25). DNA primers, deoxynucleoside triphosphate, *Taq*, and Platinum *Pfx* polymerases were purchased from Invitrogen. DNA sequences of plasmid constructs were confirmed by DNA sequencing.

Construction and confirmation of isogenic *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 1,047 to 1,472 relative to the designated *algE* coding region (168). Both PCR products were hydrolyzed by using *BamHI* and inserted into vector pGEM®-T Easy (Promega). Vector pPS856 (74) was hydrolyzed with *BamHI*. The fragment containing the *aacC1* gene (encoding gentamicin acetyltransferase) flanked by two FRT (Flp recombinase target) sites was inserted into the *BamHI* site of plasmid pGEM-TEasy:*algE*NC, resulting in plasmid pGEM-TEasy:Δ*algE*ΩGm. The 1,989 bp *algE*ΩGm comprising DNA fragment was amplified by *Pfx* polymerase using primers algE1N-Ec5 and algE2C-Ec5, and the

corresponding PCR product was inserted into *SmaI* site of vector pEX100T (74), resulting in plasmid pEX100T:Δ*algE*ΩGm.

E. coli S17-1 was used as donor for transfer of plasmid pEX100T:Δ*algE*ΩGm into *P. aeruginosa* strains, and transconjugants were selected on mineral salt medium (153) containing gentamicin and 5% (w/v) 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 algEup and algEdown.

E. coli S17-1 was used to transfer the Flp recombinase encoding vector pFLP2 (74) into *P. aeruginosa* PDO300Δ*algE*ΩGm strains and after 24 h of cultivation on PIA medium containing 5% (w/v) sucrose, gentamicin and carbenicillin-sensitive cells were analyzed by PCR for loss of the gentamicin-resistant cassette.

Complementation of isogenic *algE* deletion mutants.

For complementation of *algE* deletion mutants, the *algE* open reading frame of *P. aeruginosa* PAO1 was amplified with *Pfx* polymerase using the primers algEN(HiSDNd) and algEC(Ba). This product was then inserted into the vector pGEM®-T Easy, resulting in the plasmid pGEM-TEasy:*algE*. The *algE* fragment was released by hydrolysis with *HindIII* and *BamHI* and was inserted into *HindIII* and *BamHI* sites of broad-host-range vector pBBR1MCS-5 (24), resulting in plasmid pBBR1MCS-5:*algE*. In addition, a plasmid was constructed encoding AlgE with a 9 amino acid deletion in the highly charged putative surface exposed loop 7 region. This was constructed by amplifying the region upstream of the deletion point with *Pfx* polymerase using the primers algEN(HiSDNd) and algEDel7N(Ba) and the region downstream of the deletion point with the primers algEDel7C(Ba) and algEC(SaI). The two products were hydrolysed with *HindIII* and *BamHI* or *BamHI* and *SacI* respectively and inserted into the *HindIII* and *SacI* sites of pBBR1MCS-5 resulting in the 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 9 putative surface exposed loops. The insertions were constructed using Site-directed, Ligase-Independent Mutagenesis (SLIM) (6). Briefly, two PCR reactions were completed for each construct

using the plasmid pGEM-TEasy:*algE* as a template: one with the "F_{FLAG}" and the corresponding "R_S" primer (algEL1F_{FLAG} and algEL1R_S; algEL2F_{FLAG} and algEL2R_S; algEL3F_{FLAG} and algEL3R_S; algEL4F_{FLAG} and algEL4R_S; algEL5F_{FLAG} and algEL5R_S; algEL6F_{FLAG} and algEL6R_S; algEL7F_{FLAG} and algEL7R_S; algEL8F_{FLAG} and algEL8R_S; algEL9F_{FLAG} and algEL9R_S), and one with the F_S and corresponding R_{FLAG} primer (algEL1F_S and algEL1R_{FLAG}; algEL2F_S and algEL2R_{FLAG}; algEL3F_S and algEL3R_{FLAG}; algEL4F_S and algEL4R_{FLAG}; algEL5F_S and algEL5R_{FLAG}; algEL6F_S and algEL6R_{FLAG}; algEL7F_S and algEL7R_{FLAG}; algEL8F_S and algEL8R_{FLAG}; algEL9F_S and algEL9R_{FLAG}) (Table S1). Plasmid template was removed by hydrolysis with *DpnI*. The two PCR products were mixed in equimolar amounts and hybridisation was achieved by incubated in H-buffer (150mM NaCl, 25mM Tris, 20mM EDTA, pH 8.0) at 99°C for 3 min followed by 3 cycles of 65°C for 5 min and 30°C for 40 min. the resulting mixture was used to transform competent *E. coli* Top10 cells. Selection for cells containing the new plasmid was performed on ampicillin containing medium and plasmids were extracted. Insertion of the 24 bp FLAG encoding region was confirmed by DNA sequencing of the ORF, resulting in plasmids pGEM-TEasy:*algE*L1FLAG to pGEM-TEasy:*algE*L9FLAG. The *algE* (FLAG) containing fragments were hydrolysed and inserted in to pBBR1MCS-5 as described above, resulting in the 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, pBBRMCS-5:*algE*L9FLAG.

Alginate production assays.

2 ml of overnight culture was harvested at 4°C and washed twice with saline. Then, 200 µl of cell suspension was plated onto PIA medium and incubated for 72 h at 37°C. Cells of two agar plates were scraped off by using a sterile spatula and washed twice with 100 ml of saline (retaining the alginate containing supernatant for subsequent precipitation). Cellular sediments were freeze-dried, and the final weight was determined. Alginate supernatants were precipitated with 1 vol of ice-cold isopropanol, and 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% (w/v), followed by incubation with 15 µg of *DNaseI*/ml and 15 µg of *RNaseA*/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 further 18 h at 37°C. Solutions were dialyzed against 5 liters

of ultrapure H_2O for 48 h. Alginate was precipitated with 1 volume of ice-cold isopropanol and freeze-dried for quantification and uronic acid analysis.

Free uronic acids (alginate degradation products) were measured in the supernatant of 2 ml of overnight cultures. 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 weight cut-off 10 kDa) and the flow through was collected. The uronic acid content of the flow through (containing free uronic acids and short length alginate degradation products) was determined as described below.

Uronic acid assay.

Alginate concentrations were assayed using the uronic acid assay described previously (11), using alginic acid from brown seaweed (Sigma-Aldrich, Inc) as standard. Briefly, alginate samples were dissolved in 200 µl ultrapure H_2O at concentrations between 0.25 and 0.05 mg/ml. The sample was mixed with 1.2 ml tetraborate solution (0.0125 M disodiumtetraborate in concentrated sulphuric acid) and incubated on ice for 10 min. The mixtures were incubated at 100°C for 5 min and then cooled down on ice for further 5 min. 20 µl of m-hydroxybiphenyl reagent (0.15% m-hydroxybiphenyl in 0.125 M NaOH) was added and reactions were mixed for 1 min. For each sample or dilution a negative control was assayed using 0.0125 M NaOH instead of the m-hydroxybiphenyl reagent. 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 (1 h at 5,000*g*) and washed twice 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 followed by 15 s cool down. Cellular debris and the remaining intact cells were sedimented by centrifugation (at 5,000*g* for 1 h). The total membrane fraction was then isolated by centrifugation at 100,000*g* for 2 h. The supernatant (soluble fraction) was removed, and the sediment was resuspended in 1 volume of 10 mM HEPES containing 0.7% (w/v) *N*-lauroylsarcosine, and incubated at room temperature with shaking for 20 min to selectively solubilise the cytoplasmic

membrane. This was then centrifuged at 100,000*g* for 2 h, the resulting sediment represents the outer membrane fraction. The total protein concentration of the respective fractions was determined using the Quant-iT™ Protein Assay Kit (Invitrogen).

Analysis of outer membrane proteins.

15 µg 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 using the iBlot® dry blotting system (Invitrogen). The nitrocellulose membrane was blocked with 5% (w/v) skim milk powder in TBS with 0.1% Tween-20 for 1 h and subsequently probed with 1 µg/ml anti-FLAG (M2) tag antibody conjugated to Horseradish peroxidase (Abcam, Cambridge, UK). The membrane was washed and bound antibodies were resolved using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, U.S.A) and developed on X-ray film. Bands suspected to be AlgE or the AlgE mutants were identified by tryptic peptide fingerprinting using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

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	(77)
PDO300Δ*algE*	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	(163)
Plasmids		
pGEM®-T Easy	Ap^r, P_{lac}	Invitrogen
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	(93)
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	This study

	the ORF	
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	(74)
pEX100T:Δ*algE* ΩGm	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	(74)
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(74)

Results

Construction of an isogenic knock-out mutant of *algE*.

To investigate the requirement of AlgE in alginate biosynthesis a marker-free *algE* deletion mutant of alginate-overproducing strain *P. aeruginosa* PDO300 was generated. This mutant showed a non-mucoid phenotype when grown on solid media. Outer membrane protein profiles showed that AlgE was absent in PDO300Δ*algE* (Fig. 1A). It has been shown that the lack of mucoidity associated with some deletion mutants (*algK*, *algG* and *algX*) is distinct from the biosynthesis/polymerisation of alginate. These mutants secrete alginate degradation products (free uronic acids), shown to be the products of alginate lyase, AlgL (80-82, 143). 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). Little significant uronic acid was detected from the negative control PDO300Δ*alg8*(pBBR1MCS-5) (Table 2). These results suggest that the Δ*algE* mutant is capable of the synthesis / polymerisation of alginate but it is subsequently degraded in the periplasm.

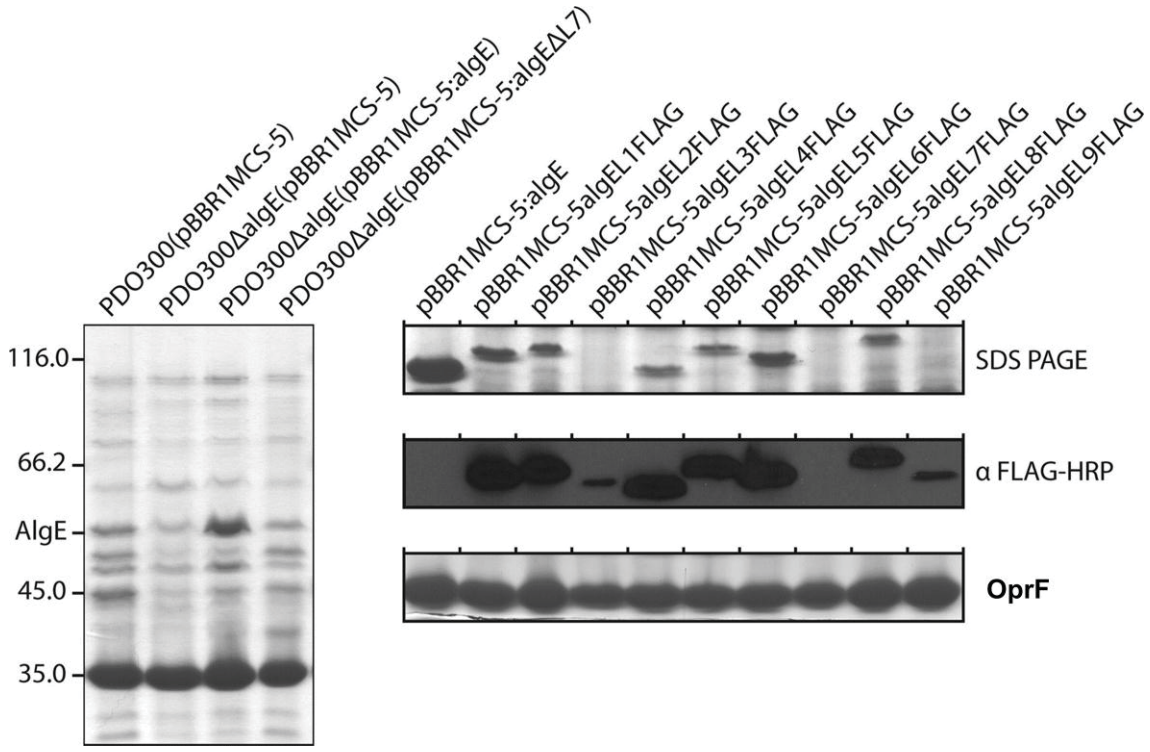

Fig. 1: Outer membrane profiles of the Δ*algE* mutant and the Δ*algE* mutant harbouring various plasmids showing the absence / presence of AlgE. (Left pannel) SDS-PAGE stained with coomassie blue shows a faint bands with the same apparent MW as AlgE present in the PDO300Δ*algE*(pBBR1MCS-5) and PDO300Δ*algE*(pBBR1MCS-5:*algE*ΔL7) outer membrane profiles were identified as Flagellin type B protein by MALDI-TOF MS. **(**Right pannel**)** Presence of tagged AlgE proteins in the outer membrane as indicated by Western immunoblot (middle). Constitutively produced OprF was used to standardize samples (bottom).

Complementation of the PDO300 Δ*algE* isogenic knock-out 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*) and 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 (5 times greater) that of the parent strain containing the vector control PDO300 (pBBR1MCS-5) (Table 2). This indicated that there were no polar effects in the deletion mutant and demonstrated the requirement of AlgE for the mucoid phenotype and the formation of intact and extracellular alginate. Interestingly, the complemented strain

PDO300Δ*algE*(pBBR1MCS-5:*algE*) produced more alginate degradation products than PDO300 (pBBR1MCS-5) (53.6% of total uronic acids compared to 8.4%) (Table 2). The presence of AlgE in the outer membrane was also restored (Fig 1A, left panel).

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:*algE*ΔL7)	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 9 extended surface exposed loops and 8 short periplasmic turns (Fig. 2). This is similar to what was predicted previously (129, 131). A search through HHpred (44) did not reveal homology to any annotated protein families or domains with known structures with which to refine this model.

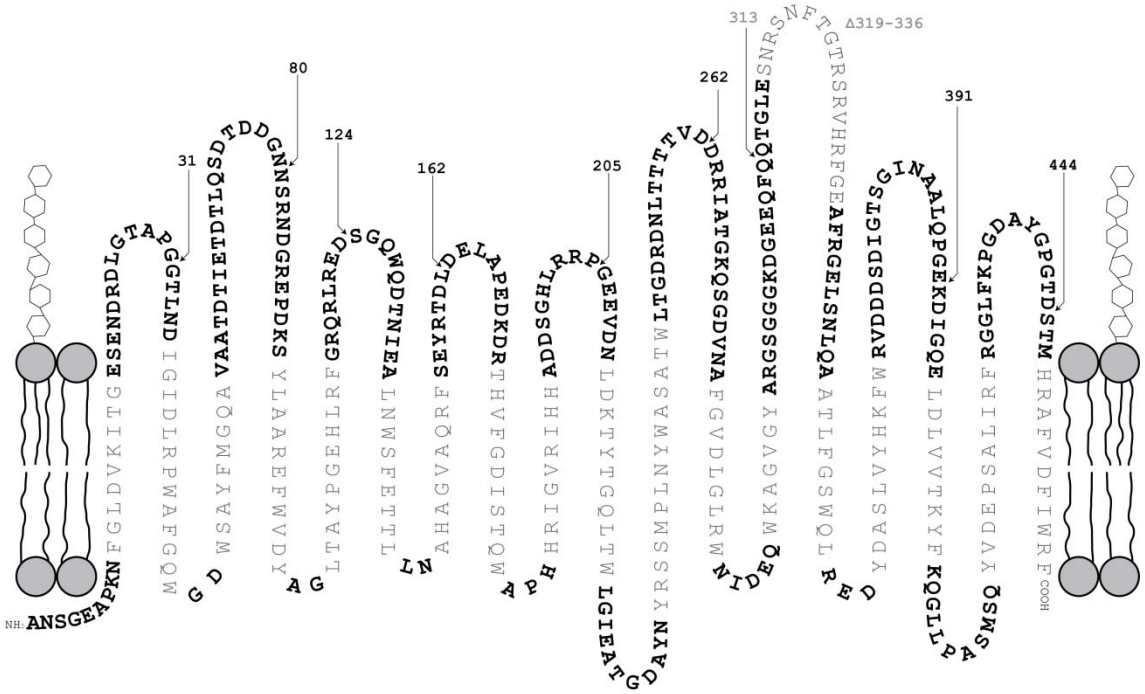

Fig. 2: Predicted topology of the outer membrane β-barrel AlgE. Predicted transmembrane β-sheets are indicated in regular text (non-boldface), extracellular loops at the top are indicated in boldface and periplasmic turns at the bottom are in boldface. Locations where the FLAG epitope were inserted are indicated by arrows and position (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, grey numbers, the insertion was non-permissive. The deleted region of loop 7 is indicated by regular grey text (non-boldface).

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 (187) (data not shown) as well as AlgJ form *A. vinelandii* (129). 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 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 levels than those of the *algE* deletion mutant, PDO300Δ*algE* (pBBR1MCS-5).

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 utilises 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 (117, 134). The FLAG epitope was inserted into the predicted loop regions for loops 1 to 9 using SLIM (24) at 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 subjected to Western inumobloting using 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 weights 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 appear to be present in significantly lower quantities than the other AlgE proteins (Fig 1B). 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).

Table 3: Alginate production of Δ*algE* complemented with various epitope tagged AlgE variants.

Strain	**Alginate (g/g CDW) ± SD**
PDO300Δ*algE*(pBBR1MCS-5:*algE*L1FLAG)	0.728 ± 0.080
PDO300Δ*algE*(pBBR1MCS-5:*algE*L2FLAG)	1.261 ± 0.236
PDO300Δ*algE*(pBBR1MCS-5:*algE*L3FLAG)	0.874 ± 0.081
PDO300Δ*algE*(pBBR1MCS-5:*algE*L4FLAG)	0.835 ± 0.067
PDO300Δ*algE*(pBBR1MCS-5:*algE*L5FLAG)	1.091 ± 0.053
PDO300Δ*algE*(pBBR1MCS-5:*algE*L6FLAG)	0.796 ± 0.146
PDO300Δ*algE*(pBBR1MCS-5:*algE*L7FLAG)	ND
PDO300Δ*algE*(pBBR1MCS-5:*algE*L8FLAG)	0.654 ± 0.105
PDO300Δ*algE*(pBBR1MCS-5:*algE*L9FLAG)	0.892 ± 0.158

[a]CDW, cellular dry weight

[b]ND – None Detected

Discussion

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 non-mucoid 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 polymerised 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 (3, 60, 80-82, 143). This supports the hypothesis that alginate is "guided" through the periplasm by a multiprotein complex or scaffold containing the periplasmic proteins AlgK, AlgX, AlgG, AlgL and the outer membrane 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 multiprotein complexes, and/or the membrane fusion domain of Alg44 (10, 138). Indeed, mutants lacking Alg44 show reduced levels of AlgE in the outer membrane (120).

The *in trans* complementation of the Δ*algE* mutant still resulted in the secretion of free uronic acids (albeit less than 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 multiprotein complexes contributing to alginate production, while the additional copy number might also lead to dysfunctional multiprotein complex formation, causing the degradation of some of the alginate as it transverses 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 sp.* in the *Pseudomonas* genome database (187) 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 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 utilised to probe the topologies of various outer membrane proteins (117, 134, 181). 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, despite the fact that 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 similar concentration of charged residues similar to that found in other eyelet loops (13, 83, 170, 178, 179). 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 does not migrate aberrantly as a 54 kDa protein as described elsewhere (26, 131), but at its deduced molecular weight 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 (134) (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-modifiability characteristics resulting in differences in their migration patterns when they are subjected to SDS-PAGE (63).

In conclusion, experimental evidence was provided for the requirement of AlgE for the secretion of alginate, whereas it is not required for alginate polymerisation and translocation into the periplasm. Furthermore, it was demonstrated that loop 7 is required for functional folding of AlgE, while the other insertion mutants support the proposed membrane topology as a β-barrel with 18 β-strand and 9 loops. Overall, the data suggest AlgE might not only be involved in the secretion of alginate but also contributes to proposed protein scaffold, guiding and protecting the nascent alginate chain through the periplasm and outer membrane.

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Supplementary Material

Table S1. Oligonucleotides used in This study.

Name	Oligonucleotides	source
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)	ACCAAAGCTTAGGAGAGAAAGCATATGAACAGCTCCCGTTCCGTCAACC	This study
algEC(Ba)	GACGGATCCTCAGAAGCGCCAGATGAAGTCGAC	This study
algEDelL7N(Ba)	TACAAGGATCCCTCCAGCCCGGTCTGCTGGAACTGCTC	This study
algEDelL7C(Ba)	AGCTAGGATCCTTCCGCGGCGAACTGAGCAACCTCCAG	This study
algEC(SaI)	GACGAGCTCTCAGAAGCGCCAGATGAAGTCGAC	This study
algEL1F$_{FLAG}$	GACTACAAGGACGACGACGACAAGGGCACCCTCAACGACATCGG	This study
algEL1R$_S$	GCCGGGAGCGGTGCCGAGGT	This study
algEL1F$_S$	GGCACCCTCAACGACATCGG	This study
algEL1R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGCCGGGAGCGGTGCCGAGGT	This study
algEL2F$_{FLAG}$	GACTACAAGGACGACGACGACAAGAACAGCCGCAACGACGGTCG	This study
algEL2R$_S$	GTTGCCGTCGTCGGTGTCCG	This study
algEL2F$_S$	AACAGCCGCAACGACGGTCG	This study
algEL2R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGTTGCCGT	This study

	CGTCGGTGTCCG	
algEL3F$_{FLAG}$	GACTACAAGGACGACGACGACAAGAGCGGC CAGTGGCAGGACAC	This study
algEL3R$_{S}$	GTCTTCCCGCAGGCGCTGGC	This study
algEL3F$_{S}$	AGCGGCCAGTGGCAGGACAC	This study
algEL3R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGTCTTCCC GCAGGCGCTGGC	This study
algEL4F$_{FLAG}$	GACTACAAGGACGACGACGACAAGGACGAA CTGGCTCCGGAGGA	This study
algEL4R$_{S}$	GAGGTCGGTGCGGTATTCGC	This study
algEL4F$_{S}$	GACGAACTGGCTCCGGAGGA	This study
algEL4R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGAGGTCG GTGCGGTATTCGC	This study
algEL5F$_{FLAG}$	GACTACAAGGACGACGACGACAAGGGCGAG GAAGTCGACAACCT	This study
algEL5R$_{S}$	GGGGCGGCGCAGGTGGCCGC	This study
algEL5F$_{S}$	GGCGAGGAAGTCGACAACCT	This study
algEL5R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGGGGCGG CGCAGGTGGCCGC	This study
algEL6F$_{FLAG}$	GACTACAAGGACGACGACGACAAGGACCGG CGCATCGCCACCGG	This study
algEL6R$_{S}$	GTCGACCGTGGTGGTGGTCA	This study
algEL6F$_{S}$	GACCGGCGCATCGCCACCGG	This study
algEL6R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGTCGACC GTGGTGGTGGTCA	This study
algEL7F$_{FLAG}$	GACTACAAGGACGACGACGACAAGCAGACC GGGCTGGAGAGCAAT	This study
algEL7R$_{S}$	CTGGAACTGCTCCTCGCCGTC	This study
algEL7F$_{S}$	CAGACCGGGCTGGAGAGCAAT	This study
algEL7R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCCTGGAAC TGCTCCTCGCCGTC	This study
algEL8F$_{FLAG}$	GACTACAAGGACGACGACGACAAGAAGGAC ATCGGCCAGGAACT	This study

algEL8R$_S$	CTCGCCCGGTTGCAGGGCGG	This study
algEL8F$_S$	AAGGACATCGGCCAGGAACT	This study
algEL8R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCCTCGCCCG GTTGCAGGGCGG	This study
algEL9F$_{FLAG}$	GACTACAAGGACGACGACGACAAGTCGACC ATGCACCGCGCCTT	This study
algEL9R$_S$	GTCGGTGCCCGGCCCGTAGG	This study
algEL9F$_S$	TCGACCATGCACCGCGCCTT	This study
algEL9R$_{FLAG}$	CTTGTCGTCGTCGTCCTTGTAGTCGTCGGTG CCCGGCCCGTAGG	This study

Chapter IV

The dual roles of *Pseudomonas aeruginosa* AlgE in secretion of the virulence factor, alginate, and formation of the secretion complex

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Abstract

AlgE is a monomeric 18-stranded β-barrel protein required for secretion of the extracellular polysaccharide, alginate, in *Pseudomonas aeruginosa.* To assess the molecular mechanism of alginate secretion, AlgE was subjected to site-specific and FLAG epitope insertion mutagenesis. Except for β-strands 6 and 10, epitope insertions into the transmembrane β-strands abolished localization of AlgE to the outer membrane. Interestingly, an epitope insertion into β-strand 10 produced alginate and was only detectable in outer membranes isolated from cells grown on solid media. The deletion of nine C-terminal amino acid residues destabilised AlgE. Replacement of amino acids which constitute the highly electropositive pore constriction showed that individual amino acid residues have a specific function in alginate secretion. Two of the triple mutants K47E/R353A/R459E and R74E/R362A/R459E severely reduced alginate production. Mutual stability analysis using the *algE* deletion mutant PDO300Δ*algE*(miniCTX) showed that the periplasmic alginate biosynthesis proteins AlgK and AlgX were completely destabilised while the copy number of the inner membrane c-di-GMP receptor Alg44 was reduced. Chromosomal integration of *algE* restored AlgK, AlgX and Alg44 providing evidence for a multiprotein complex that spans the cell envelope. Periplasmic turn 4 of AlgE was identified as important region for maintaining the stability of the putative multiprotein complex.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen of particular relevance to cystic fibrosis (CF) patients whose lungs are susceptible to severe and chronic infections by the bacterium. In the CF lung *P. aeruginosa* converts to a highly mucoid phenotype, which is characterized by the overproduction of alginate (72). Alginate serves as an extracellular matrix component enabling the formation of differentiated biofilms that confer resistance to antibiotics, and prevents phagocytosis by the immune system of the host (54, 123). Alginate is an unbranched random polymer consisting of β-D-mannuronic acid and its C-5 epimer α-L-guluronic acid. Most of the genes required for alginate biosynthesis are located on a single operon under control of the *algD* promoter (23). Products of these genes are involved in precursor biosynthesis, polymerisation, modification and secretion (48, 130). Alginate precursor biosynthesis also requires AlgC, the gene for which is located outside this operon. AlgC is also involved in rhamnolipid and lipopolysaccharide biosynthesis (121). The precursor for alginate biosynthesis, GDP mannuronic acid, is synthesized from fructose-6-phosphate by the concerted actions of AlgA, AlgC and AlgD (72). Polymerization of alginate requires two inner membrane proteins, Alg8 and Alg44 (138, 139). Alg8 is predicted to have multiple membrane spanning regions and a large cytoplasmic glycosyltransferase domain, while Alg44 has one transmembrane domain, a cytoplasmic PilZ c-di-GMP binding domain and a periplasmic domain which is predicted to be similar to the membrane fusion protein, MexA from the MexAB-OprM multi-drug efflux pump (108, 120, 138). The nascent polymannuronate chain is believed to enter the periplasmic space into a scaffold formed by AlgK, G, X, L, 44, E, and the proteins involved in alginate acetylation AlgJ, I and F. AlgK is an outer membrane lipoprotein with multiple copies of the tetratricopeptide-repeat protein-protein interaction motif (86). AlgK has been demonstrated to interact with AlgX, which in turn interacts with MucD, a serine protease involved in regulation of alginate biosynthesis (71). Deletion of *algK, algE* or *algX* results in secretion of free uronic acids, which are degradation products of high molecular weight alginate (61, 69, 81, 110, 143). The alginate lyase, AlgL, is thought to play a dual role in alginate biosynthesis, both as a component of the putative periplasmic scaffold and degrading mislocalized periplasmic alginate (3, 82). Free uronic acids are believed to be secreted when the integrity of the scaffold/complex is compromised and alginate escapes into the periplasm and is exposed to the alginate

lyase, AlgL. AlgG is an acetylation sensitive epimerase that converts non-acetyled D-mannuronate to its C-5 epimer L-guluronate at the polymer level (46). Alginate secreted by *P.aeruginosa* can also be selectively O-acetylated at the O2' and/or O3' positions of mannuronate residues by the actions of AlgI, AlgJ and AlgF (51). The recent structure of AlgE reveals that it is a monomeric outer membrane 18-stranded β-barrel porin (184). An isogenic deletion mutant of *algE* has shown that AlgE is essential for alginate production (69), but the presence of free uronic acids indicates that not only is AlgE required for the secretion of full-length alginate but that the protein may also play a role in the formation of the periplasmic scaffold/complex. The periplasmic turns of AlgE have been suggested as potential sites for mediating its interaction with the other periplasmic components required for alginate biosynthesis (184). The AlgE pore is lined with highly conserved, charged amino acid residues, which have been suggested to confer selectivity towards alginate and/or facilitate its efficient secretion across the outer membrane (184).

In the present study, a site-specific mutagenesis approach, guided by the structure of AlgE, was applied to assess the role the membrane segments, periplasmic turns and pore constricting residues play in alginate secretion and the stability, subcellular localization, and assembly of the proposed multiprotein periplasmic complex. Chromosomal AlgE variants, which contain FLAG epitope insertions in the periplasmic turns, have also been used to gain insight into the proposed interaction(s) between AlgE and other components of the periplasmic scaffold.

Material and Methods

Construction of FLAG epitope insertion variants.

The FLAG epitope was inserted into seven transmembrane segments and six periplasmic turns by site-directed, ligase independent mutagenesis (24). In brief, two PCR products were made for each FLAG epitope insertion using pGEM-TEasy:*algE* as template. One with primer pair "F_{FLAG}" and the corresponding reverse primer "Rs" (*e.g.* algEM2F_{FLAG} and algEM2Rs) and the second with primer pair "Fs" and corresponding reverse primer "R_{FLAG}" (*e.g.* algEM2Fs and algEM2R_{FLAG}) (Table S1). An AlgE variant, AlgEtrC9, with nine amino acids removed from the C-terminus was created using primers "algEN-HiSDNd" and reverse primers "algECtr9". Plasmid template was removed at the end of the PCR by addition of 10 µl of D-buffer (20 mM $MgCl_2$, 20 mM Tris pH 8.0, 5 mM DTT) containing 10 units of *Dpn*I and incubating the mixture at 37°C for 60 min. The *Dpn*I treated products were mixed for the hybridisation reaction with 10 µl of 5X H-buffer (750 mM NaCl in 125 mM Tris pH 9.0, and 100 mM EDTA pH 8.0 with the final pH of 8.5), 15 µl of each PCR product and autoclaved water to bring the volume to 50 µl. The hybridization mixture was incubated at 99°C for 3 min followed by two cycles of 65°C for 5 min and 30 °C for 15 min. 20 µl of hybridized product was used to transform *Escherichia coli* competent TOP 10 cells. Selection of cells containing the new plasmid was achieved on LB plates containing ampicillin at a concentration of 75 µg/ml of media. The insertion of the 24 bp FLAG epitope was confirmed by sequencing the ORF. The resulting pGEM-TEasy plasmids containing the different *algE*-(FLAG) insertions were hydrolysed with *BamHI* and *HindIII* and the resulting 1497bp fragments were ligated into pBBR1MCS-5 to produce the following plasmids: pBBR1MCS-5:*algE*M2FLAG; pBBR1MCS-5:*algE*M4FLAG; pBBR1MCS-5:*algE*M6FLAG; pBBR1MCS-5:*algE*M8FLAG; pBBR1MCS-5:*algE*M10FLAG; pBBR1MCS-5:*algE*M12FLAG, pBBR1MCS-5:*algE*M14FLAG; pBBR1MCS-5:a*lgE*T2FLAG; pBBR1MCS-5:*algE*T4FLAG; pBBR1MCS-5:*algE*T5FLAG, pBBR1MCS-5:*algE*T6FLAG; pBBR1MCS-5:*algE*T8-1FLAG; pBBR1MCS-5:*algE*T8-2FLAG; pBBR1MCS-5:*algE*T8-3FLAG; pBBR1MCS-5:*algE*Ctr9. The pBBR1MCS-5 carrying variants of *algE* were transferred into PDO300Δ*algE* by electroporation and selecting the transformants on Pseudomonas Isolation Agar (PIA) plates containing 300 µg/ml of gentamycin (Table S2).

Generation of site-specific variants.

Site-directed mutants of AlgE were created as described above and resulted in the construction of plasmids: pBBR1MCS-5:*algE*K47E; pBBR1MCS-5:*algE*R74E; pBBR1MCS-5:*algE*R129E; pBBR1MCS-5:*algE*R154E; pBBR1MCS-5:*algE*R353A; pBBR1MCS-5:*algE*H364A; pBBR1MCS-5:*algE*R362A; pBBR1MCS-5:*algE*R365A; pBBR1MCS-5:*algE*R459E; pBBR1MCS-5:*algE*R461E; pBBR1MCS-5:*algE*R152A; pBBR1MCS-5:*algE*N164A; pBBR1MCS-5:*algE*R481E; pBBR1MCS-5:*algE*E130A; pBBR1MCS-5:*algE*D162A; pBBR1MCS-5:*algE*E189A; pBBR1MCS-5:*algE*D193A; pBBR1MCS-5:*algE*E368A; pBBR1MCS-5:*algE*D485A. Using pGEM-TEasy:*algE*R459E as a template, double amino acid substitution variants pGEM-TEasy:*algE*R353A+R459E, pGEM-TEasy:*algE*R362A+R459E and pGEM-TEasy:*algE*E368A+R459E were made. The pGEM-Teasy plasmids containing different site-directed mutants of *algE* were hydrolysed with *BamHI* and *HindIII* and the resulting 1473bp fragments ligated into pBBR1MCS-5 resulting in plasmids: pBBR1MCS-5:*algE*R353A+R459E, pBBR1MCS-5:*algE*R362A+R459E and pBBR1MCS-5:*algE*E368A+R459E. To make triple mutants, plasmids pBBR1MCS-5:*algE*K47E and pBBR1MCS-5:*algE*R74E were hydrolysed with *HindIII* and *EcoRI* and the resulting 407bp fragments ligated into *HindIII* and *EcoRI* digested pBBR1MCS-5:*algE*R353A+R459E, pBBR1MCS-5:*algE*R362A+R459E and pBBR1MCS-5:*algE*E368A+R459E, resulting in plasmids pBBR1MCS-5:*algE*K47E+ +R353A+R459E, pBBR1MCS-5:*algE*R74E+R353A+R459E, pBBR1MCS-5:*algE* K47E+R362A+R459E, pBBR1MCS-5:*algE*R74E+R362A+R459E+ +, pBBR1MCS-5:*algE*K47E+E368A+R459E and pBBR1MCS-5:*algE*R74E+E368A+R459E. The pBBR1MCS-5 plasmids carrying different site-directed variants of *algE* were transferred into PDO300Δ*algE* through electroporation (Table S2). All of the restriction enzymes were purchased from Roche.

Alginate quantification.

Bacterial cultures were grown overnight and cells from 2 ml of bacterial culture were harvested and washed twice with sterile saline buffer. 200 µl of cells were spread on a PIA plate and incubated at 37°C for 72 h. Cells were scraped off the plates and washed twice with sterile saline solution while keeping the supernatant with dissolved alginate for subsequent precipitation. Cells pellets were freeze-dried and the final weight determined. Supernatant with dissolved alginate was precipitated with 1 volume of ice-

cold isopropanol and alginate was harvested and freeze-dried. For further purification alginate was dissolved in buffer A (50 mM Tris-HCl-10 mM $MgCl_2$, pH 7.4) to a final concentration of 0.5% (w/v). After alginate was solubilised in buffer A 15 µg/ml of *DNAse* and *RNAse* was added and the solution incubated at 37°C for 6 h with shaking. *Pronase E* was added to a final concentration of 20 µg/ml and the solution incubated again for 18 h at 37°C in a shaking incubator. The final solution was dialysed against 5 l of miliQ water for 48 h at 4°C in tubing with a molecular weight cut off of 12 kDa (ZelluTrans, ROTH®). After dialysis, the alginate was precipitated against one volume of ice-cold isopropanol and freeze-dried for subsequent uronic acid quantification.

The amount of free uronic acid in 2 ml of overnight grown culture was measured. Cells were pelleted down by centrifugation and the supernatant was filtered through vivaspin-500 (GE Healthcare) centrifugal filter devices with a molecular weight cut-off of 10 kDa and the flow through collected. The uronic acid content of the flow through, which contains the free uronic acids and short-chain alginate degradation products was determined as described below.

Uronic acid assay.

Alginate quantification was performed using the uronic acid assay as described previously (11). Alginic acid from brown seaweed was used as a standard (Sigma-Aldrich, Inc). Briefly, alginate samples were dissolved in MiliQ water at concentrations of between 0.25 to 0.05 mg/ml. 200 µl of these samples were mixed with 1.2 ml of tetraborate solution (12.5 mM disodium tetraborate in concentrated sulphuric acid) and incubated on ice for 10 min. This mixture was incubated at 100°C for 5 min and then cooled on ice for 5 min. 20 µl of m-hydroxybiphenyl reagent (0.15% (w/v) hydroxybiphenyl in 125 mM NaOH) was added to the reaction mixture and vortexed for 1 min. For each sample or dilution, a negative control was assayed using 125 mM NaOH instead of using hydroxybiphenyl reagent. The uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

Isolation of whole envelope and OMs.

Strains of *P. aeruginosa* were grown overnight in LB medium with appropriate antibiotics. Cells were harvested by centrifugation at 6000*g* for 30 min at 4°C and washed twice with equal volume of 10 mM HEPES pH 7.4 buffer. Cells were suspended in 10 ml of 10 mM HEPES buffer with 1 Complete Mini-EDTA-free

protease inhibitor cocktail tablet (Roche) and sonicated on ice for 12 cycles with 15 s of sonication followed by 15 s of cool down on ice. Cellular debris and unbroken cells was removed by centrifugation at 8000 *g* for 45 min at 4°C. The whole envelope fraction was isolated by centrifugation at 100,000*g* for 1 h at 4°C and washed. To isolate the outer membranes, the envelope fraction was resuspended in 1 volume of 10 mM HEPES buffer containing 0.7% (w/v) *N*-laurosyl-sarcosine and the suspension was incubated at room temperature for 20 min with shaking to solubilize the inner membrane. This mixture was centrifuged at 100,000*g* for 1 h. The pellet was resuspended in 10 ml of 10 mM HEPES buffer and centrifuged again at 100,000*g* to remove residual detergent. The resulting sediments represent the OM fraction. The total protein concentration of each respective fraction, envelope and OM, was determined using a Quant-iT protein assay kit (Invitrogen).

Analysis of outer membrane proteins.

25 µg of total protein was loaded and separated by SDS PAGE using 8% polyacrylamide gels. The resulting gels were either stained with coomassie blue stain or western blotted using the iBlot® dry blotting system (Invitrogen). After blotting, the nitrocellulose membrane was blocked with 5 % (w/v) skim milk in Tris-buffered-saline containing 0.05% (v/v) Tween-20 for 1 h at room temperature. Anti-Alg44 (1:10,000), anti-AlgK(1:10,000), anti-AlgG(1:1000), anti-AlgX(1:7000) and anti-AlgE (1:5000) antibodies, raised in rabbits against the respective purified proteins, were used as primary antibodies and anti-IgG anti-rabbit antibodies, labelled with HRP (Abcam, Cambridge, UK), used as secondary antibodies. The membrane was washed three times and bound antibodies were resolved with SuperSignal West Pico chemiluminescent substrate (Thermoscientific, Rockford, IL) and developed on X-ray film (KODAK, Rochester, New York).

Chromosomal integration.

The promoter region -879 bp relative to the *algD* open reading frame was amplified by forward (PalgPstIF) and reverse primers (PalgHindIIIR). After A-tailing, the fragment was ligated into vector pGEM®-T Easy (Promega) and the fidelity of the sequence verified. The promoter region of *algD*, 879bp, was hydrolysed from pGEM-TEasy using *PstI* and *HindIII*. Various variants of *algE* (T2F, T4F, T5F, T6F, M6F, T8F-1) and wild type *algE* were hydrolyzed using *HindIII* and *BamHI* from the respective

pGEM-TEasy backbones. Purified *algD* promoter region and individual *algE* fragments were ligated together into the integration proficient mini-CTX-*lacZ* based plasmid, hydrolysed with *PstI* and *BamHI*, to generate the miniCTX:P*algE*T2F, miniCTX:P*algE*T4F, miniCTX:P*algE*T5F, miniCTX:P*algE*T6F and miniCTX:P*algE* plasmids. These plasmids were then transferred into PDO300Δ*algE* strains by electroporation and selected for on media containing tetracycline at a concentration of 150 µg/ml. Integration into the chromosome was confirmed through PCR using PserUP and PserDOWN primers (75). The backbone of mini-CTX-*lacZ* plasmid was removed by transferring the pFLP2 plasmid through electroporation and subsequently pFLP2 was cured by cultivating for 24 h on MSM media containing 5% (w/v) sucrose (154). Tetracycline and carbenicillin sensitive cells were analysed by PCR to confirm the removal of mini-CTX-*lacZ* backbone. Similarly mini-CTX-*lacZ* plasmid was transferred into PDO300 and PDO300Δ*algE* and confirmed by PCR.

Results

Mutational analysis of residues involved in the secretion of alginate.

Site-directed mutagenesis of AlgE was performed to identify amino acid residues that are critical for alginate secretion. Highly conserved amino acid residues that line the pore of the β-barrel, constrict the channel opening, and contribute to its electropositive surface were selected, as these residues may confer specific binding and/or selectivity towards its polyanionic substrate, alginate (184). To assess the role of these residues single, double and triple amino acids substitution variants of AlgE were created. Residues K47, R74, R129, R152, D162, N164, R353, R362, R459, D485, were selected as they are highly conserved and constrict the channel. Most of these residues were found conserved in *P. aeruginosa*, *P. putida*, *P. entomophilia*, *P. mendocina*, *P. fluorescence*, *P. syringae* and *Azotobacter vinelandii* (184).To reduce the positive electrostatic field inside the AlgE lumen, positively charged arginine and lysine residues were substituted with glutamate and alanine, while asparagine and negatively charged aspartate residues were replaced with alanine (Fig. 1A, 1B).

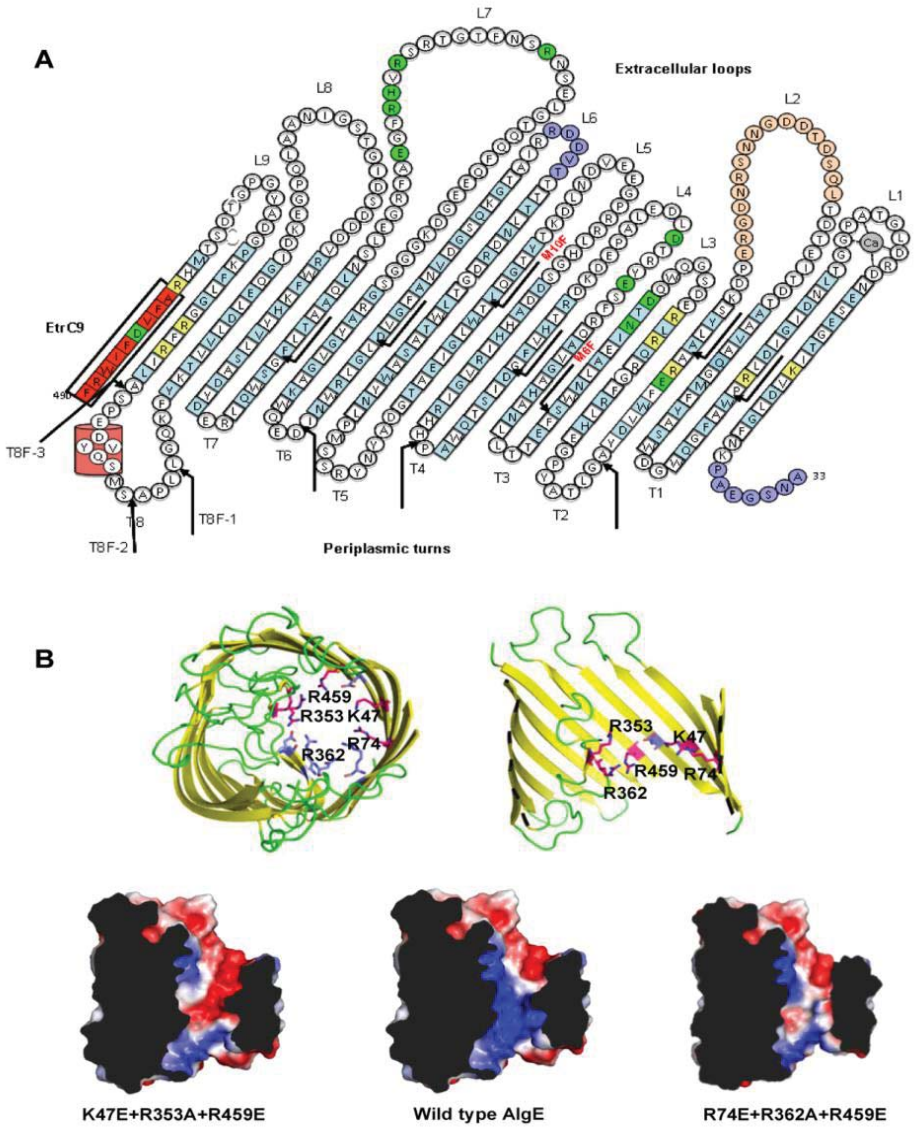


Fig. 1: (A) Membrane topology of AlgE showing FLAG epitope insertion sites and mutated residues. (B) Triple mutations targeting the pore constriction of AlgE decrease the positive electrostatic field within the β-barrel lumen. (A) Topology model of AlgE based on the X-ray crystal structure (modified from Whitney, J. C. *et al* (2011)) (184). Arrows indicate the positions at which FLAG epitopes were inserted. "EtrC9" indicates the location of residues 482-490, which were deleted at the C-terminus of the protein. Amino acid residues forming the transmembrane β-sheet are given in squares. Amino acid residues are represented by single letter code. Amino acid residues with side chains pointing into the barrel lumen are coloured blue. Alanine and glutamic acid substituted amino acids residues are coloured green and yellow, respectively. The amino acid residues which could not be modelled in the crystal structure were coloured blue-purple. Ca in L1 represents a calcium ion**.** (A) Cartoon representation of AlgE as shown from the extracellular surface (upper left) and in the

plane of the outer membrane (upper right) with the top exposed to the cell surface and the bottom exposed to the periplasm. In each model, the pore-forming residues are indicated in sticks representation. Residues colored in pink, K47E, R74E, R353A, R459E and R362A, constitute residues in the two triple mutants with significantly reduced alginate production. Electrostatic surface representation of wild type AlgE and the AlgE K47E/R353A/R459E and R74E/R362A/R459E triple mutants (Lower panel). Note that the positive electrostatic field (blue) inside the putative alginate translocation pathway is replaced by a negative electrostatic field (red) in the both triple mutants. Mutated residues were modelled as their lowest energy rotamers in COOT. Electrostatics were generated in Pymol.

The amount of secreted alginate mediated by these AlgE variants was quantified. Except for R129A, D162A, and D485A mutation of each of the selected amino acid residues, resulted in reduced production of alginate when compared to the wild type (Fig. 2).

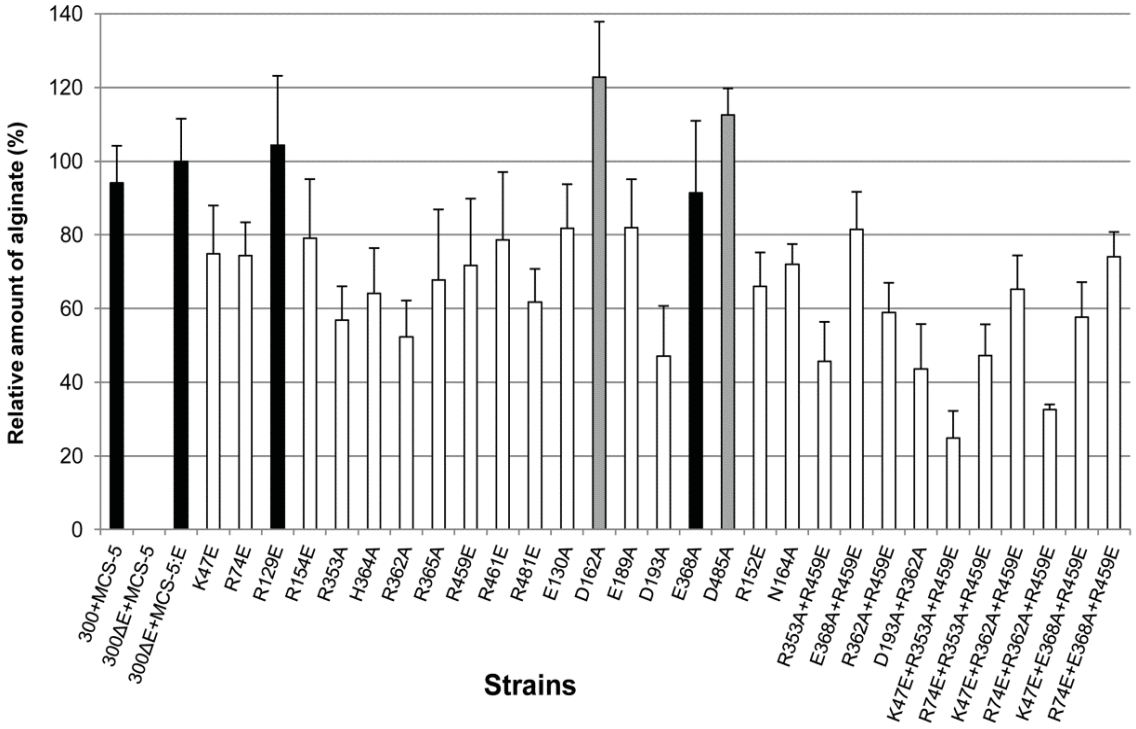


Fig. 2: Relative amount of alginate produced by *P. aeruginosa* PDO300Δ*algE* harbouring various plasmids. The amount of alginate (normalised to the amount of AlgE in OM) produced by the PDO300Δ*algE* complemented with *algE* site specific variants is presented relative to the amount produced by *P. aeruginosa* PDO300Δ*algE* complemented with wild type *algE*. Amount of alginate produced by wildtype AlgE strain given as 100% corresponds to 0.729g/g of CDW. Quantification of bands was performed by using Gel Analysis Software UN-SCAN-IT gel 6.1 (Silkscientific). Experiments were conducted in triplicates and the error bars represent the standard deviation of the mean value. An unpaired student T-test was applied and $P \leq 0.05$ was considered as significant. Variants with no significant change in alginate production are coloured black, those show showing increase in alginate production are coloured gray while those showing a reduction in alginate production are coloured white. 300+MCS-5 indicates PDO300 carrying pBBR1MCS-5 plasmid; 300ΔE+MCS-5 shows PDO300Δ*algE* carrying pBBR1MCS-5 plasmid; 300ΔE+MCS-5:E depicts PDO300Δ*algE* carrying pBBR1MCS-5:*algE* plasmid. Variants of AlgE are designated by the single letter code and number for each amino acid in wild type AlgE that was targeted, followed by single letter code of the amino acid that the residue was mutated to.

OMs derived from PDO300Δ*algE* expressing the various mutated *algE* genes were subjected to SDS-PAGE and western blot analysis, using primary anti-AlgE antibodies. All site-specific mutations were structurally tolerated and the respective AlgE variants were able to localize to the OM (Fig. 3).

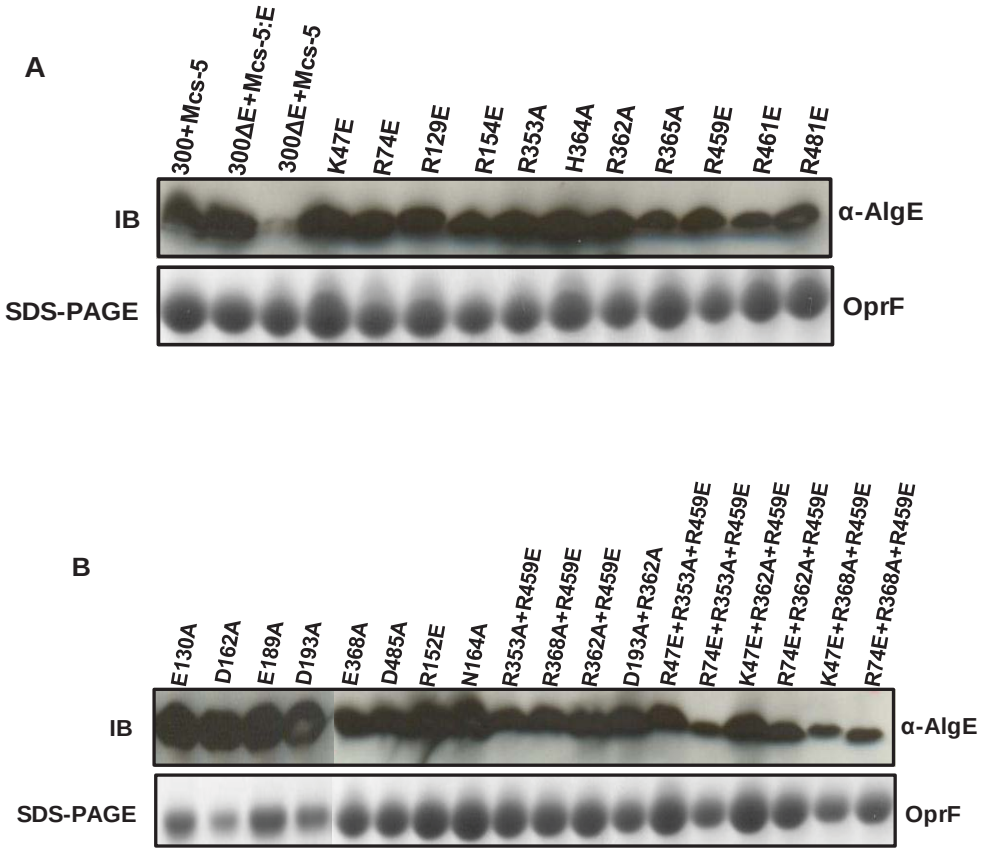


Fig. 3: Outer membrane localization of AlgE and its variants. Outer membranes from planktonic cultures of *P. aeruginosa* PDO300Δ*algE* and *P. aeruginosa* PDO300Δ*algE* harbouring various plasmids encoding AlgE were isolated and analysed by immunoblotting (IB). Constitutively expressed OprF was used as a loading control (bottom panel). Only the relevant parts of the gels and the immunoblot are shown.

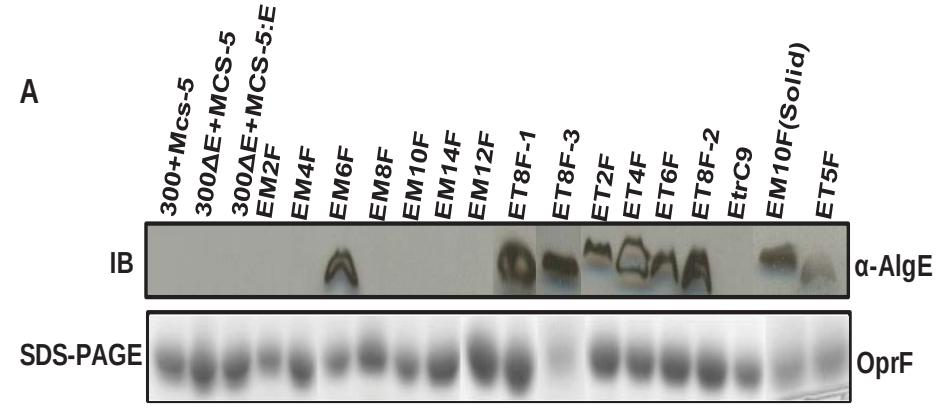
It has previously been shown that deletions or insertions in loop L7, located between β-strands 13 and 14, were not tolerated and completely ablated alginate production (69). The structure of AlgE revealed that this loop is folded inside the lumen of the β-barrel, where it restricts the size of the pore opening and helps stabilize the structure (184). We therefore replaced amino acids R353, R362, H364, R365 and E368 with alanine to further explore the role of loop L7 in defining the specificity of the pore and AlgE function. Mutation of R353, R362, H364 and R365 resulted in at least a 30% reduction in alginate production, while the E368A variant did not show any significant difference in alginate production (Fig. 2). Substitutions were also made to other residues which line the inside of the AlgE pore (E130A, R154E, E189A, D193A, R461E, R481E) and various double (R353A+R459E, E368A+R459E, R362A+R459E, D193A+R362A) and triple mutants (K47E+R353A+R459E, R74E+R353A+R459E, K47E+R362A+R459E, R74E+R362A+R459E, K47E+E368A+R459E, R74E+E368A+R459E) were created. All of these variants showed a decrease in the amount of alginate produced compared to wild type AlgE. The largest reductions in alginate production were observed for the triple mutants, K47E+R353A+R459E and R74E+R362A+R459E, which showed 75% and 70% decrease in alginate production, respectively, compared to wild type AlgE (Fig. 2).

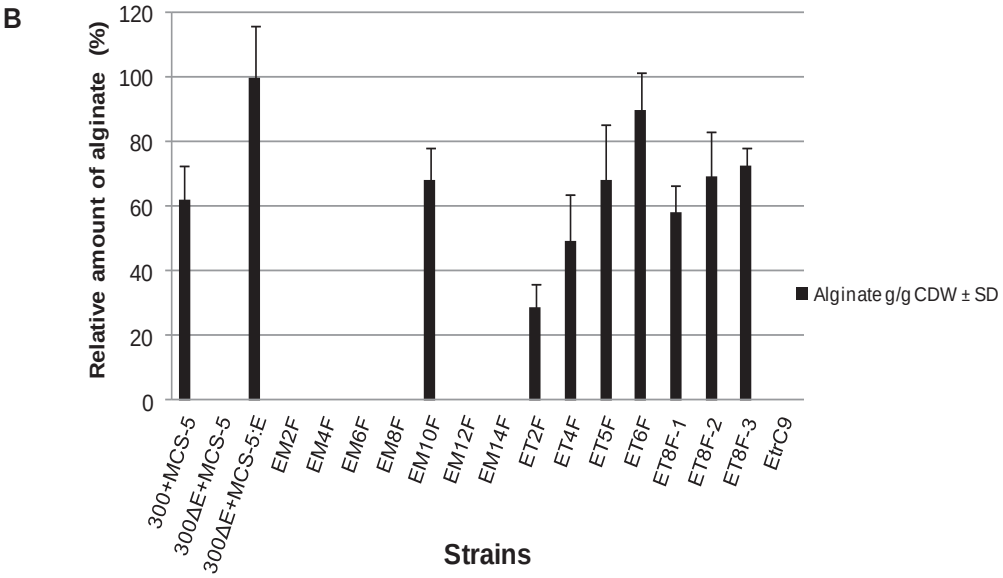
Structure-function analysis using FLAG epitope insertion mutagenesis.

To assess the structural and functional importance of different regions of AlgE, FLAG epitopes were inserted in transmembrane regions and periplasmic turns using a site-directed mutagenesis approach (24). FLAG epitopes were inserted in alternating β-strands starting from β-strand 2 through to β-strand 14 and also in selected periplasmic turns to generate the AlgE variants EM2F, EM4F, EM6F, EM8F, EM10F, EM12F and EM14F, and ET2F, ET4F, ET5F, ET6F, ET8F-1, ET8F-2 and ET8F-3, repectively (Fig. 1A). These variants were used to investigate the role of these regions in protein folding and stability, and the ability of the periplasmic turns to interact with other periplasmic components of the alginate synthesis machinery. The selection of the periplasmic turns was based on the AlgE structure. Plasmids encoding these variants of AlgE were then introduced into PDO300Δ*algE* and their subcellular localization and ability to restore alginate production assessed. Variants of AlgE with FLAG epitope insertions in β-strands 2, 4, 8, 12 and 14 (EM2F, EM4F, EM8F, EM12F and EM14F) were unable to localize to the OM, when cells were grown in either planktonic mode or on solid media,

as shown by their absence in the OM fractions analysed by immunoblotting (Fig. 4A). These variants were unable to restore alginate production to PDO300Δ*algE* (Fig. 4B) and produced 100% free uronic acids (Fig. 4C). Insertion in β-strand 6 (EM6F) was tolerated with respect to subcellular localisation to the OM but was not functional as no high molecular weight alginate was detected (Fig. 4A, 4B). This variant produced 100% free uronic acids (Fig. 4C). The EM10F variant of AlgE with the FLAG epitope inserted in β-strand 10 was the only β-strand insertion tested that was capable of restoring alginate production (Fig. 4B). While the EM10F variant could not be detected in the OM when cells were grown in liquid media, it was detected in OMs isolated from cells grown on solid media (Fig. 4A) and is therefore consistent with our alginate quantification assay which measures the amount of alginate produced after growth of the cells on solid media.

Insertions of FLAG epitopes in the periplasmic turns were tolerated as judged by their insertion into the OM and the restoration of alginate production (Fig. 4A, 4B). AlgE variants with insertions in periplasmic turns T2, T4, T5 and T8-1 produced 100% free uronic acids in liquid culture (Fig. 4C). Variants of AlgE with insertions in periplasmic turns T6, T8-2, T8-3 produced 70-80% free uronic acids when compared to the *algE* deletion mutant (Fig. 4C). The ET6F variant produced the most alginate. The levels of both alginate and uronic acids produced by this variant were comparable to the amounts produced by the *algE* deletion mutant complemented with the wild type *algE* gene (Fig. 4B, 4C). Deletion of nine amino acids at the C-terminus of AlgE abolished localization to the OM and alginate production (Fig. 4A, 4B) and produced 100% free uronic acids (Fig. 4C).


A
300+Mcs-5
300ΔE+MCS-5
300ΔE+MCS-5:E
EM2F
EM4F
EM6F
EM8F
EM10F
EM14F
EM12F
ET8F-1
ET8F-3
ET2F
ET4F
ET6F
ET8F-2
EtrC9
EM10F(Solid)
ET5F
IB
α-AlgE
SDS-PAGE
OprF


B
Relative amount of alginate (%)
120
100
80
60
40
20
0
300+MCS-5
300ΔE+MCS-5
300ΔE+MCS-5:E
EM2F
EM4F
EM6F
EM8F
EM10F
EM12F
EM14F
ET2F
ET4F
ET5F
ET6F
ET8F-1
ET8F-2
ET8F-3
EtrC9
Strains
Alginate g/g CDW ± SD

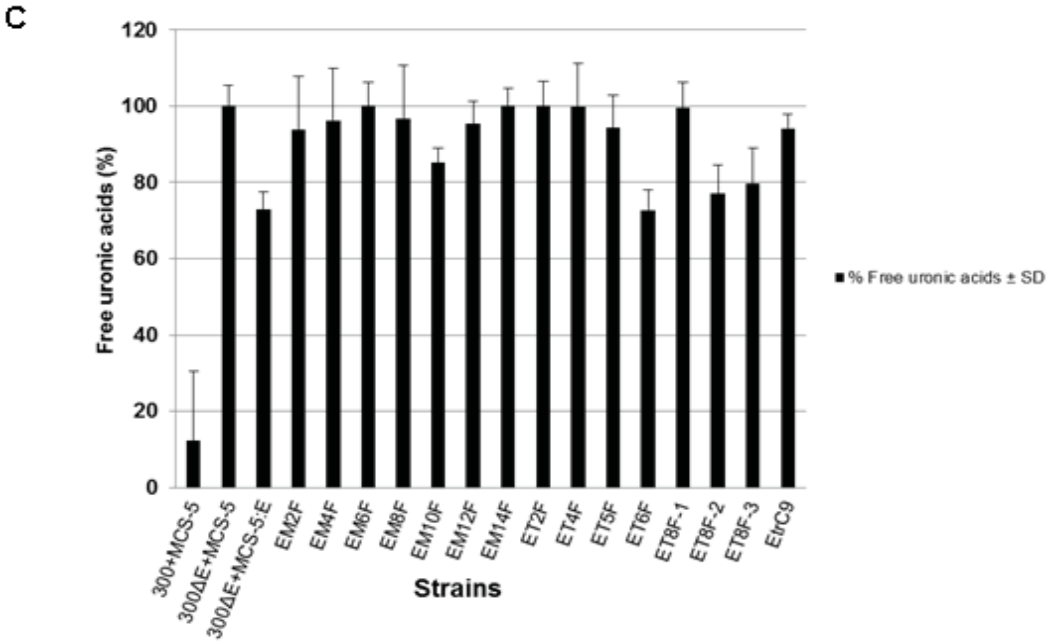

C
Free uronic acids (%)
120
100
80
60
40
20
0
300+MCS-5
300ΔE+MCS-5
300ΔE+MCS-5:E
EM2F
EM4F
EM6F
EM8F
EM10F
EM12F
EM14F
ET2F
ET4F
ET5F
ET6F
ET8F-1
ET8F-2
ET8F-3
EtrC9
Strains
% Free uronic acids ± SD

Fig.4: Localization, alginate and free uronic acid production by FLAG epitope insertion variants of AlgE. (A) OM fraction isolated from planktonic cultures (unless mentioned from solid) of the *P. aeruginosa* PDO300Δ*algE* harbouring various plasmids showing the absence or presence of AlgE and its variants. Presence of AlgE and its variants in the OM can be seen in the immunoblots (IB) probed with anti-AlgE antibodies (upper panel). EM10F (solid) indicates that the OM was isolated from PDO300Δ*algE*(pBBR1MCS-5:*algE*M10F) cells grown on solid media. Constitutively expressed OprF was used as a loading control (bottom panel). Only the relevant parts of the gels and the immunoblots were shown. (B) The amount of alginate produced was assessed by growing cells on solid media and is given relative to the amount of alginate produced by *P. aeruginosa* PDO300Δ*algE* (pBBRMCS-5:*algE*). (C) The amount of free uronic acid was assessed after overnight growth in liquid culture and is given as the percentage of ratio between the filterate and the supernatant. Experiments were conducted in triplicates. Error bars show the standard deviation (SD) of the mean value. 300+MCS-5 indicates PDO300 carrying pBBR1MCS-5 plasmid; 300ΔE+MCS-5 shows PDO300Δ*algE* carrying pBBR1MCS-5 plasmid; 300ΔE+MCS-5:Edepicts PDO300Δ*algE* carrying pBBR1MCS-5:*algE* plasmid. Variants of AlgE are indicated as first letter E indicating wild type AlgE followed by M for membrane segment or T for periplasmic turn, followed by the number indicating their position in AlgE as defined by the structure and shown schematically in Fig. 1. The letter F is used as an abbreviation for FLAG epitope.

Mutual stability effect of AlgE and its variants on components of the proposed periplasmic scaffold.

It has been proposed that the alginate biosynthesis machinery forms a supra-molecular complex (140), and that AlgE interacts with AlgK (86, 184). As deletion of AlgE might affect the stability of other components of the alginate biosynthesis machinery, we integrated wild type *algE* and its variants encoding FLAG insertions in periplasmic turns T2, T4, T5 and T6 into the chromosome. The ET2F, ET4F, ET5F were selected as they produce 100% free uronic acids during planktonic growth suggesting a role in stabilising alginate biosynthesis complex. The ET6F variant was used as FLAG epitope control. OMs and envelope fractions were isolated, and the OMs subjected to immunoblot analysis using an anti-AlgE antibody (Fig. 5). Wild type AlgE and all the variants tested were detected in the OM, in agreement with the results we observed in our *in trans* complementation studies (Fig. 4A). The AlgE variant (ET2F) was present in lower copy number, while as expected, AlgE was absent from the OM of PDO300Δ*algE*(miniCTX) (negative control) (Fig. 5). The ET2F variant was detected at wild type levels in OMs isolated from cells grown on solid media (data not shown). Envelope fractions isolated from all the strains were probed with primary anti-AlgK, anti-AlgX, anti-AlgG and anti-Alg44 (rabbit) antibodies. In envelope fractions isolated from PDO300Δ*algE*(miniCTX) AlgK and AlgX were not detected and the protein amount of Alg44 was reduced relative to PDO300Δ*algE*(miniCTX:*algE*) (Fig. 5).

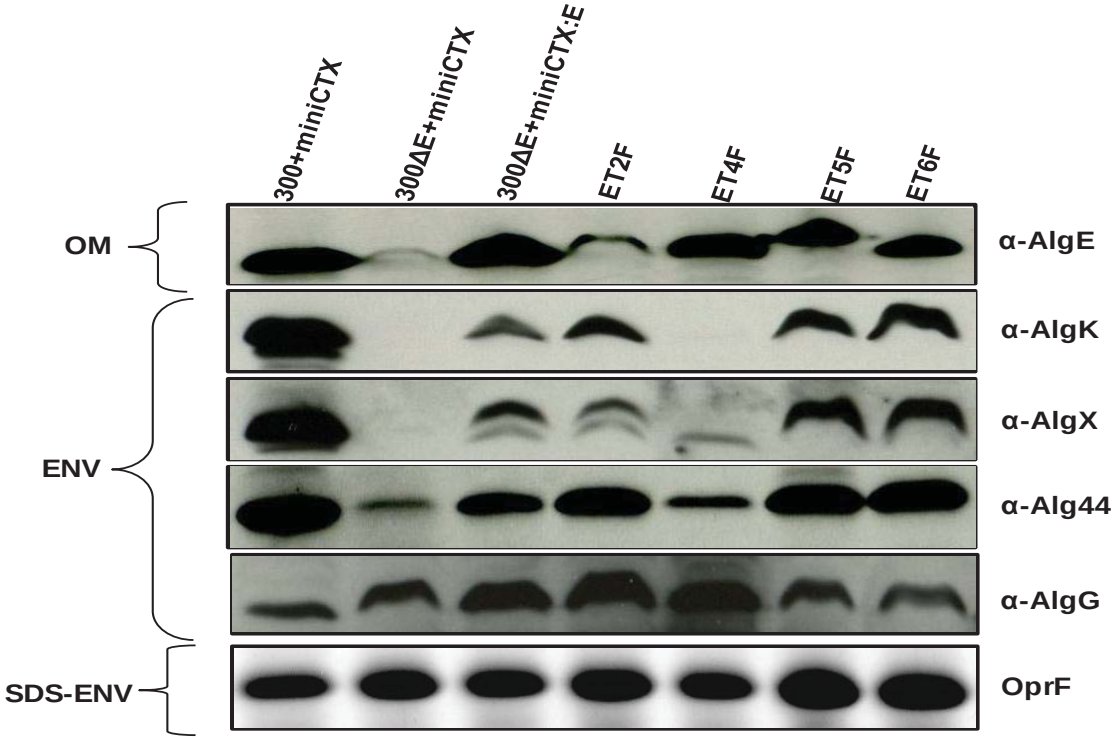


Fig. 5: Effect of AlgE and its variants on the stability of other components of alginate biosynthesis machinery. Genes encoding AlgE and its variants were integrated into the chromosome of *P. aeruginosa* PDO300Δ*algE* using the mini-CTX-*lacZ* integration plasmid. Outer membranes and envelope fractions were isolated and immunoblotting was performed using specific primary antibodies as indicated. OM: outer membrane, ENV: Envelope fraction, SDS-ENV: SDS-PAGE of envelope fraction. 300+miniCTX indicates PDO300 carrying integration proficient miniCTX plasmid; 300ΔE+miniCTX shows PDO300Δ*algE* carrying miniCTX plasmid; 300ΔE+miniCTX:E depicts PDO300Δ*algE* carrying miniCTX:*algE* plasmid. Variants of AlgE are indicated as first letter E indicating wild type AlgE followed by M for membrane segment or T for periplasmic turn, followed by the number indicating their position in AlgE as defined by the structure and shown schematically in Fig. 1. The letter F is used as an abbreviation for FLAG epitope. Constitutively expressed outer membrane protein OprF was used as a loading control.

Expression of *algE in trans* in PDO300Δ*algE*(pBBR1MCS-5:*algE*) did not restore the presence of AlgK or AlgX possibly because of a disturbance in stoichiometry of proteins (data not shown). However chromosomally integrated *algE* and variants (ET2F, ET5F, ET6F) restored AlgK, AlgX and Alg44 (Fig. 5). Interestingly, the chromosomally integrated variant of AlgE, ET4F, destabilised AlgK and AlgX, to the point that AlgK could not be detected. In this variant the copy number of Alg44 appeared to be reduced while AlgX is present as a lower molecular weight band suggesting potentially proteolytic degradation (Fig. 5). A small reduction in the level of AlgG was observed in PDO300Δ*algE*(miniCTX) and AlgE variants ET5F and ET6F as compared to PDO300Δ*algE*(miniCTX:*algE*) (Fig. 6). Chromosomal integration of *algE* and the T2F, T4F, T5F, and T6F variants restored alginate production to varying extents and produced varying amounts of free uronic acids (Fig. 6A, 6B).

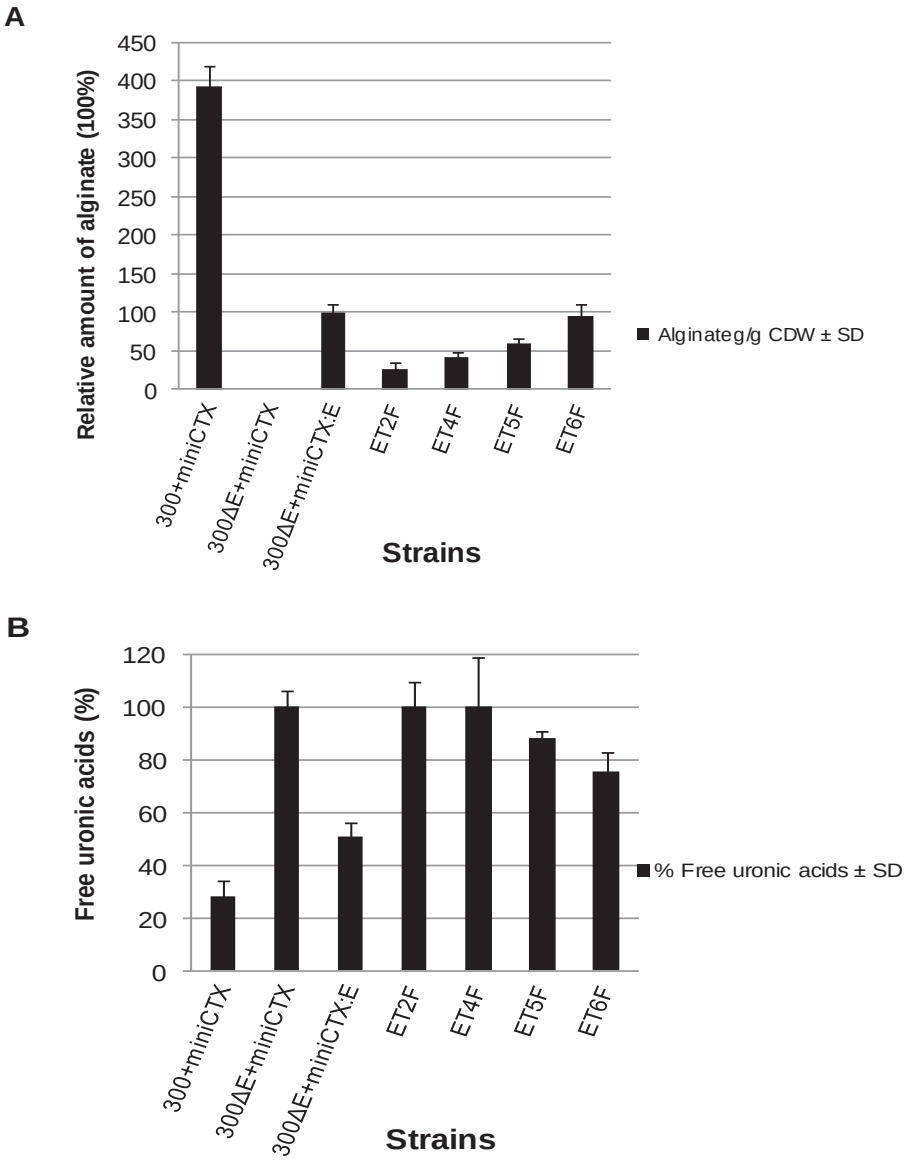


Fig. 6: Alginate and free uronic acids produced by *P. aeruginosa* with chromosomal integration of genes encoding AlgE and its variants. (A) The amount of alginate is shown relative to the amount of alginate produced by PDO300Δ*algE* (miniCTX:*algE*). (B) The amount of free uronic acids is given as a percentage of the ratio between filtrate and supernatant. Experiments were conducted in triplicates and the error bars represent the standard deviation of the mean value. 300+miniCTX indicates PDO300 carrying integration proficient miniCTX plasmid; 300ΔE+miniCTX shows PDO300Δ*algE* carrying miniCTX plasmid; 300ΔE+miniCTX:E depicts PDO300Δ*algE* carrying miniCTX:*algE* plasmid. Variants of AlgE are indicated as first letter E indicating wild type AlgE followed by M for membrane segment and T for periplasmic turn which is followed by the number indicating their respective position from the N-terminus of AlgE and F stands for FLAG epitope insertion.

Discussion

OM proteins are synthesized in the cytoplasm and secreted into the periplasm through Sec translocons (39). After cleavage of the N-terminal signal sequence in the periplasm, the mature proteins are sequestered by periplasmic chaperones, which transport them to the β-barrel assembly machinery (BAM) with its core component BamA located in the OM. The BAM machinery, with the aid of the periplasmic chaperones, fold and insert β-barrel proteins into the OM (89). This machinery is responsible for the correct folding and insertion of AlgE in the outer membrane. The structure of AlgE reveals that the inside of its β-barrel is highly positively charged (Fig. 1A, 1B), as might be expected for the selection and/or efficient secretion of negatively charged alginate.

The proposed conduit for alginate secretion is lined with the highly conserved charged residues K47, R74, R129, R152, D162, N164, R353, R362, R459 and D485 as well as the less well conserved residues E130, R154, E189, D193, H364, R365, E368, R461 and R481 (184). Except for R129, mutation of each of the positively charged residues resulted in a decrease in the amount of alginate produced, while the replacement of the negatively charged D485 and D162 showed an increase in alginate production (Fig. 3). Substitutions of R353, R362, H364 and R365 located on loop 7 led to reduced alginate production suggesting an important role of this loop not only in protein stability, as previously described (69), but also in alginate secretion (184). The identification of a role for loop 7 in substrate recognition is not unique to AlgE, as other substrate specific porins, *e.g.* OccD1, OccK1 (41), and LamB, have residues located on comparable channel constriction loops that define substrate specificity in these systems. To further investigate the impact of site-directed mutantagenesis on the function of AlgE double and triple variants were created. While all of these double and triple amino acids substitution variants showed a decrease in alginate production, two triple mutants, K47E+R353A+R459E and R74E+R362A+R459E, showed the largest decrease in alginate production (Fig. 2). In these triple mutations the positively charged arginine (R) and lysine (K) residues that line the constriction channel were substituted with either glutamic acid (E) or alanine (A) residues. These substitutions would result in a decrease in the net positive charge and an increase in the net negative charge inside the lumen (Fig. 1B). The increased electronegative potential might interfere with selection and/or efficient secretion of the negatively charged substrate, alginate. This is further supported by the observation that substitution of negatively charged D162 or E485 with alanine,

increased alginate production (Fig. 2). All of the site-specific variants of AlgE were detected in the OM (Fig. 3).

AlgE was originally described as a general diffusion protein but recently it has been proposed to form a substrate specific pore (62, 184). The substrate specificity is supported by the observation that the structure of AlgE superimposes with other substrate specific porins of *P. aeruginosa* such as OccD1 and OccK1 with rms deviation of 2.7Å and 2.9Å over 302 and 306 equivalent Cα positions, respectively (184). The OccD family of proteins is involved in the uptake of small molecular weight substrates, a highly divergent function compared to the proposed function of AlgE. The electropositive conduit (8Å in diameter) of AlgE also suggests its specific for alginate (184). Our data shows that changing the conduit to a more electronegative potential impacts alginate production and hence suggests that AlgE exhibits properties as a substrate specific porin. As perhaps expected, none of the AlgE site-specific or multiple point variants show a complete loss of functionality. This is similar to results obtained previously for the substrate specific maltoporin, LamB, where replacement of all six residues found to be involved in carbohydrate transport was required to abolish substrate binding (36). The secretion of alginate by site-specific AlgE variants is further explained by the fact that sugar-protein interactions are generally weak, and thus even in the triple mutants some alginate would still be secreted by the force of alginate polymerisation.

To study the structural and functional importance of different regions of AlgE in AlgE stability, protein-protein interaction and secretion of alginate we inserted FLAG epitopes into transmembrane regions and periplasmic turns. Transmembrane β-strands are amphipathic in nature and disruption of this amphipathicity by inserting a foreign epitope can affect the protein's ability to fold and insert into the OM. interestingly AlgE variant M6F, was detected in OM (Fig. 4A). The loss of alginate, but not uronic acid, (Fig. 4B, 4C) production by this variant suggests that the FLAG insertion at this site, three amino acids upstream of the periplasmic turn 3 (Fig. 1A), does not affect the ability of the nine residues upstream to form β-strand 6. In this case the FLAG epitope and three residues downstream of it would be pushed into the periplasm, and thus could result in a large and strongly charged periplasmic turn 3 which in turn could interfere with the ability of the AlgE variant to secrete alginate properly. Of the FLAG epitope

insertions into the transmembrane regions, the EM10F variant is unique in that it was only detected in OMs isolated from solid media (Fig. 4A). This is consistent with the alginate quantification, which is done from solid media (Fig. 4B). It has been shown previously that alginate is overproduced by *P. aeruginosa* when grown in the biofilm mode (73). Growth conditions which favour alginate production might require upregulation of *algE* gene expression and/or more efficient assembly of the alginate biosynthesis/secretion machinery. This could have led to the presence of detectable copy numbers of the AlgE variant M10F.

The production of 100% free uronic acids by ET2F, ET4F, ET5F, ET8F-1 during planktonic growth of cells harbouring the respective plasmids *i.e.* multiple copies of each gene (Fig. 4C) suggested that FLAG epitope insertions in these periplasmic turns might interfere with the ability of AlgE to interact with other proteins of the alginate biosynthesis machinery. Previously it has been shown that deletion of the large periplasmic turn, T8, resulted in the production of 50% free uronic acids when the respective AlgE variant was encoded by a plasmid (184). This suggested that insertion of the FLAG epitope in turn 8 (ET8F-1) could interfere with AlgE's function by locking T8 into an open conformation, destabilising the neighbouring turns and interfering with the ability of AlgE to interact with periplasmic scaffold proteins, thus causing destabilisation of the alginate polymerisation/secretion multiprotein complex which resulted in production of 100% free uronic acid. The T8 deletion variant was not considered for integration into the chromosome because the deletion of T8 produced 50% free uronic acids and the possibility that ET8F-1 could have destabilised a neighbouring turn. Future studies would try to explain the exact function of T8 in alginate secretion and biosynthesis.

It has been proposed that hydrophobic residues at the C-terminus of β-barrel porins, especially the terminal residue, play a role in targeting these proteins to the OM. For example, the C-terminal F of PhoE was shown to be required for efficient localisation of the protein to the OM (169). AlgE has a comparable signature sequence of W-R-F at its C-terminus, and thus it perhaps not surprising that truncation of the last nine residues, EtrC9, results in complete loss of the protein (Fig. 4A, 4,B, 4C).

The alginate biosynthesis machinery is proposed to span the entire envelope fraction and to form a multiprotein complex. This multiprotein complex is proposed to guide the

alginate through the periplasm to AlgE. This assumes that components of this machinery are interacting with each other at least when alginate is produced. Indeed it has been proposed that AlgK, which contains at least 9.5 tetratricopeptide-like protein-protein interaction motifs interacts with AlgE (86, 184). Experimental data have suggested an interaction between AlgK with AlgX, and AlgX and the periplasmic serine protease, MucD, a negative regulator of alginate biosynthesis (60, 71). Our assessment of the impact of AlgE and its FLAG insertion variants on the stability of other components of alginate biosynthesis machinery suggests that AlgE interacts with AlgK (Fig. 5). As an OM lipoprotein, AlgK is likely to be in close proximity to AlgE, and since deletion of AlgK caused mis-localization of AlgE, the data presented herein also support a direct interaction between these two components (86). Destabilisation of the periplasmic component AlgX could be an indirect consequence of destabilization of AlgK, which interacts with AlgX. Our results do not allow us to determine whether AlgE is interacting directly with Alg44, the proposed co-polymerase. For Alg44 to interact directly with AlgE it would require Alg44 to have a large periplasmic domain that is capable of spanning the entire periplasmic region (~200Å) (33). Based on the homology models of Alg44, it thus seems more likely that Alg44 interacts with AlgX and/or AlgK. These results support the hypothesis that a multiprotein complex that spans the entire envelope region is required for alginate secretion (140). Polymerization of alginate has been previously shown to require components present in both the inner and outer membranes (61).

Two variants of AlgE, ET5F and ET6F, also showed a slight reduction in the level of AlgG suggesting that periplasmic turns T5 and T6 could play a role in stabilising AlgG through either a direct or indirect interaction (Fig. 5). Interestingly, the AlgE variant with an insertion in periplamic turn 4 (ET4F), when grown under planktonic conditions, completely destabilised AlgK, significantly reduced the amount of full-length AlgX and reduced the copy number of Alg44 (Fig. 5). This result, like that observed for the EM10F variant, suggests a difference in expression of the alginate operon or stability of respective alginate biosynthesis protein when bacteria are grown in liquid culture and solid media, as this variant produces ~45% full length alginate relative to the wild type protein when alginate production was analyzed in solid media (Fig. 6A). This level of alginate production would not be expected given the complete destabilization of AlgK and the production of 100% uronic acids in an *algK* deletion mutant (81). Interestingly,

AlgE variant ET4F did not affect the stability of AlgK, AlgX and Alg44 when cells were harvested from solid media (unpublished data). However our results presented here are consistent with free uronic acids analysis being done from liquid culture. The T4 turn is the smallest of all the periplasmic turns (only two amino acids protruding into the periplasm) tested in this study (Fig. 1A) and while there is a discrepancy between the solid and liquid media results, they do suggest that any protein possibly interacting with this turn should localize close to the OM; a criteria that could be fulfilled by AlgK.

Based on the findings in this study a model has been generated showing the potential involvement of AlgE in stabilizing members of the periplasmic scaffold via specific protein-protein interactions (Fig. 7). Additional studies aimed at providing experimental evidence for the existence of a multiprotein complex by identifying direct protein-protein interactions are in progress.

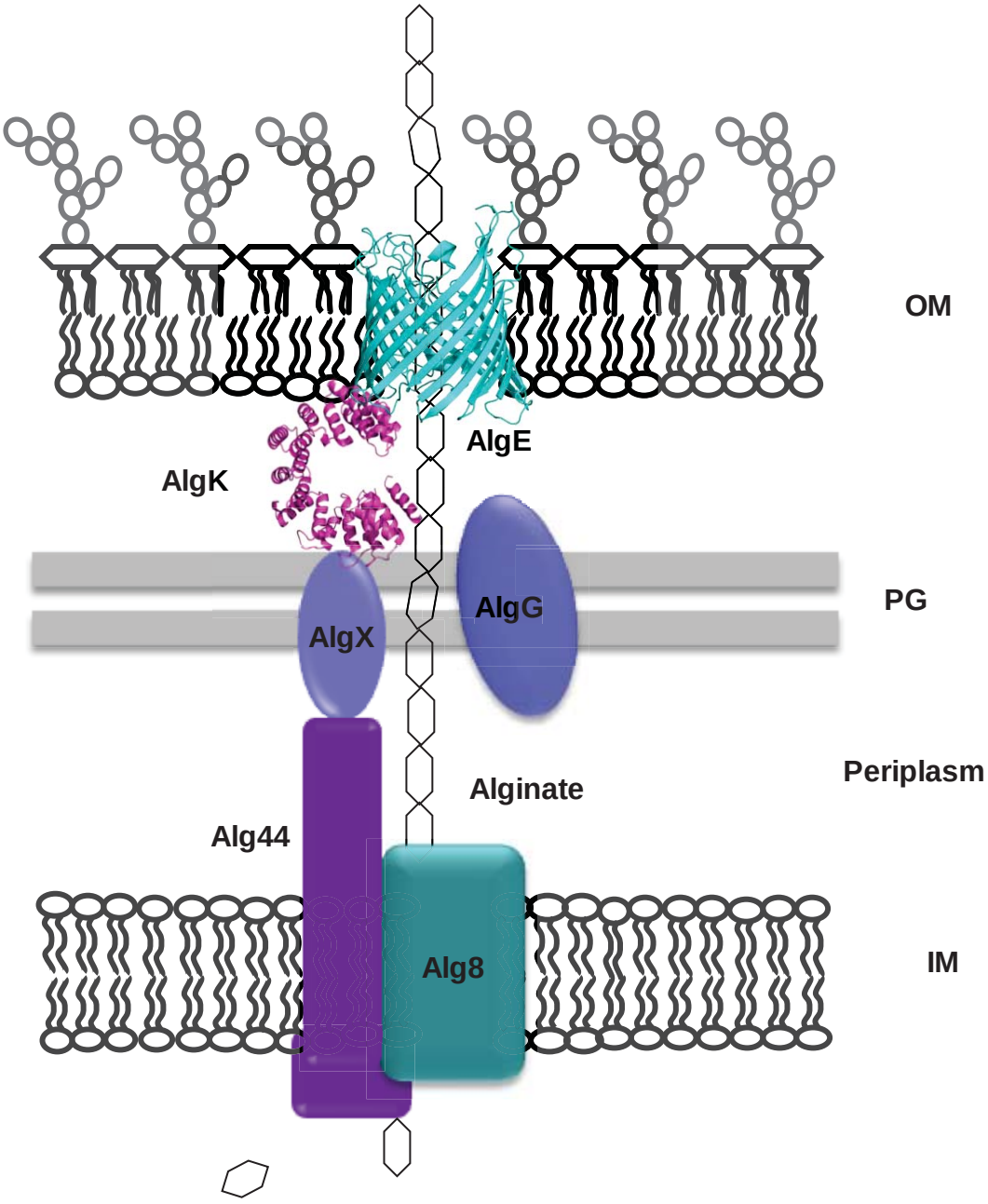


Fig. 7: Model of proposed AlgE interactions based on our mutual stability results. The protein-protein interactions depicted above are based on mutual stability analysis using AlgE and its variants, and result from Hay, I. D. *et al* (71). The analysis of the impact of FLAG-tag variants of AlgE on the mutual stability of other components of the alginate biosynthesis machinery suggests that AlgK may interact with periplasmic turns 4 of AlgE, although we cannot rule out the involvement of other periplasmic turns. Alginate is represented as a chain of hexagons. OM, outer membrane; IM, inner membrane; PG, peptidoglycan.

Acknowledgements

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Supplementary Material

Table S1.Oligonucleotides used in the study.

Name	Primer sequence	Source
AlgEM2Ft	GACTACAAGGACGACGACGACAAGTGGGCCTTCGGCCAGTGGGG	This study
AlgEM2Fs	TGGGCCTTCGGCCAGTGGGG	
AlgEM2Rt	CTTGTCGTCGTCGTCCTTGTAGTCGGGCCGCAGGTCGATACCGA	
AlgEM2Rs	GGGCCGCAGGTCGATACCGA	
AlgEM4Ft	GACTACAAGGACGACGACGACAAGGCACGCGAATTCTGGGTCGA	This study
AlgEM4Fs	GCACGCGAATTCTGGGTCGA	
AlgEM4Rt	CTTGTCGTCGTCGTCCTTGTAGTCGGCGAGGTAGCTCTTGTCCG	
AlgEM4Rs	GGCGAGGTAGCTCTTGTCCG	
AlgEM6Ft	GACTACAAGGACGACGACGACAAGTTCGAGACCACCCTGCTCAA	This study
AlgEM6Fs	TTCGAGACCACCCTGCTCAA	
AlgEM6Rt	CTTGTCGTCGTCGTCCTTGTAGTCGCTCCAGTTCAGCGCCTCGA	

AlgEM6Rs	GCTCCAGTTCAGCGCCTCGA	
AlgEM8Ft	GACTACAAGGACGACGACGACAAGGACATTTCCACCCAGTGGGC	This study
AlgEM8Fs	GACATTTCCACCCAGTGGGC	
AlgEM8Rt	CTTGTCGTCGTCGTCCTTGTAGTCGCCGAACACATGGGTGCGGT	
AlgEM8Rs	GCCGAACACATGGGTGCGGT	
AlgEM10Ft	GACTACAAGGACGACGACGACAAGACCTGGCTCGGCATCGAGGC	This study
AlgEM10Fs	ACCTGGCTCGGCATCGAGGC	
AlgEM10Rt	CTTGTCGTCGTCGTCCTTGTAGTCGAGCTGGCCGGTATAGGTCT	
AlgEM10Rs	GAGCTGGCCGGTATAGGTCT	
AlgEM12Ft	GACTACAAGGACGACGACGACAAGCTCGGCCTGCGCTGGAACAT	This study
AlgEM12Fs	CTCGGCCTGCGCTGGAACAT	
AlgEM12Rt	CTTGTCGTCGTCGTCCTTGTAGTCGTCGACGCCGAAGGCATTGA	
AlgEM12Rs	GTCGACGCCGAAGGCATTGA	
AlgEM14Ft	GACTACAAGGACGACGACGACAAGGGCTCCTGGCAACTGCGCGA	This study

AlgEM14Fs	GGCTCCTGGCAACTGCGCGA	
AlgEM14Rt	CTTGTCGTCGTCGTCCTTGTAGTCGAACAGG GTGGCTGCCTGGA	
AlgEM14Rs	GAACAGGGTGGCTGCCTGGA	
AlgET2Ft	GACTACAAGGACGACGACGACAAGGGCCTC ACCGCCTACCCCGG	This study
AlgET2Fs	GGCCTCACCGCCTACCCCGG	
AlgET2Rt	CTTGTCGTCGTCGTCCTTGTAGTCGGCGTAGT CGACCCAGAATTCG	
AlgET2Rs	GGCGTAGTCGACCCAGAATTCG	
AlgET4Ft	GACTACAAGGACGACGACGACAAGCACCAC CGCATAGGCGTGCG	This study
AlgET4Fs	CACCACCGCATAGGCGTGCG	
AlgET4Rt	CTTGTCGTCGTCGTCCTTGTAGTC CGGCGCCCACTGGGTGG	
AlgET4Rs	CGGCGCCCACTGGGTGG	
AlgET5Ft	GACTACAAGGACGACGACGACAAGTATCGT TCGAGCATGCCGCTG	This study
AlgET5Fs	TATCGTTCGAGCATGCCGCTG	
AlgET5Rt	CTTGTCGTCGTCGTCCTTGTAGTCGTTGTAGG CATCGCCGGTAGC	
AlgET5Rs	GTTGTAGGCATCGCCGGTAGC	

AlgET6Ft	GACTACAAGGACGACGACGACAAGGACGAG CAATGGAAGGCCGG	This study
AlgET6Fs	GACGAGCAATGGAAGGCCGG	
AlgET6Rt	CTTGTCGTCGTCGTCCTTGTAGTC GATGTTCCAGCGCAGGCCG	
AlgET6Rs	GATGTTCCAGCGCAGGCCG	
AlgET8F-1Ft	GACTACAAGGACGACGACGACAAGCTGCCG GCCTCGATGAGCC	This study
AlgET8F-1Fs	CTGCCGGCCTCGATGAGCC	
AlgET8F-1Rt	CTTGTCGTCGTCGTCCTTGTAGTCCAGGCCTT GCTTGAAGTACTTGG	
AlgET8F-1Rs	CAGGCCTTGCTTGAAGTACTTGG	
AlgET8F-2Ft	GACTACAAGGACGACGACGACAAGTCGATG AGCCAGTACGTCGA	This study
AlgET8F-2Fs	TCGATGAGCCAGTACGTCGA	
AlgET8F-2Rt	CTTGTCGTCGTCGTCCTTGTAGTCGGCCGGC AGCAGGCCTTGCT	
AlgET8F-2Rs	GGCCGGCAGCAGGCCTTGCT	
AlgET8F-3Ft	GACTACAAGGACGACGACGACAAGGCGCTG ATCCGCTTCCGCGG	This study
AlgET8F-3Fs	GCGCTGATCCGCTTCCGCGG	
AlgET8F-3Rt	CTTGTCGTCGTCGTCCTTGTAGTCCGAGGGC	

	TCGTCGACGTACTGG	
AlgET8F-3Rs	CGAGGGCTCGTCGACGTACTGG	
algEECtr9	GGATCCTCAGCGGTGCATGGTCGAGTCG	This study
AlgEN-HisDNd	ACCAAAGCTTAGGAGAGAAAGCATATGAAC AGCTCCCGTTCCGTCAACC	(69)
K47EF	GAGATCACCGGCGAATCG	This study
K47EF(SR)	CACGTCCAGGCCGAAGTTC	
K47ER	GATCTCCACGTCCAGGCC	
K47ER(SF)	ACCGGCGAATCGGAAAACG	
R74EF	CTGGAGCCCTGGGCCTTCG	This study
R74EF(SR)	GTCGATACCGATGTCGTTG	
R74ER	CTCCAGGTCGATACCGATGTC	
R74ER(SF)	CCCTGGGCCTTCGGCCAGTG	
R129EF	GCAGAGGAATTCTGGGTCGAC	This study
R129EF(SR)	GGCGAGGTAGCTCTTGTCC	
R129ER	CTCTGCGGCGAGGTAGCTC	
R129ER(SF)	GAATTCTGGGTCGACTACGC	

R459EF	ATCGAGTTCCGCGGCGGCCTG	This study
R459EF(SR)	CAGCGCCGAGGGCTCGTCGAC	
R459ER	CTCGATCAGCGCCGAGGGCTC	
R459ER(SF)	TTCCGCGGCGGCCTGTTCAAG	
R154EF	ATCGAGTTCCGCGGCGGCCTG	This study
R154EF(SR)	CAGCGCCGAGGGCTCGTCGAC	
R154ER	CTCGATCAGCGCCGAGGGCTC	
R154ER(SF)	TTCCGCGGCGGCCTGTTCAAG	
R461EF	TTCGAGGGCGGCCTGTTCAAG	This study
R461EF(SR)	GCGGATCAGCGCCGAGGGCTC	
R461ER	CTCGAAGCGGATCAGCGCCGA	
R461ER(SF)	GGCGGCCTGTTCAAGCCGGGC	
R481EF	CACGAGGCCTTCGTCGACTTC	This study
R481EF(SR)	CATGGTCGAGTCGGTGCCCGG	
R481ER	CTCGTGCATGGTCGAGTCGGT	
R481ER(SF)	GCCTTCGTCGACTTCATCTGG	
R362AF	GGCACCCGCTCGGCCGTGCACCGCTT	This study

R362AF(SR)	GGTGAAGTTGGAGCGATTG	
R362AR	AAGCGGTGCACGGCCGAGCGGGTGCC	
R362AR(SF)	CGGCGAAGCCTTCCGCGG	
R353AF	CCGGGCTGGAGAGCAATGCCTCCAACTTCAC	This study
R353AF(SR)	TCTGCTGGAACTGCTCCTC	
R353AR	TGAAGTTGGAGGCATTGCTCTCCAGCCCGG	
R353AR(SF)	CGGCACCCGCTCGCGCGTG	
R365AF	CTCGCGCGTGCACGCCTTCGGCGAAGCC	This study
R365A(SR)	CGGGTGCCGGTGAAGTTG	
R365AR	GGCTTCGCCGAAGGCGTGCACGCGCGAG	
R365A(SR)	TTCCGCGGCGAACTGAGC	
H364AF	CGCTCGCGCGTGGCCCGCTTCGGCGA	This study
H364AF(SR)	GGTGCCGGTGAAGTTGG	
H364AR	CGCCGAAGCGGGCCACGCGCGAGCG	
H364AR(SF)	AGCCTTCCGCGGCGAAC	
E368AF	CCGCTTCGGCGCCGCCTTCCGCG	This study
E368AF(SR)	TGCACGCGCGAGCGGGTG	
E368AR	CGCGGAAGGCGGCGCCGAAGCGG	

E368AR(SF)	GCGAACTGAGCAACCTCC	
E130AF	CGCGCCTTCTGGGTCGACTAC	This study
E130AR	GGCGCGTGCGGCGAGGTAGCT	
D162AF	CAGGCCACCAACATCGAGGCG	This study
D162AR	GGCCTGCCACTGGCCGCTGTC	
E189AF	AGCGCCTACCGCACCGACCTC	This study
E189AR	GGCGCTGAAACGCTGGGCGAC	
D193AF	ACCGCCCTCGACGAACTGGCT	This study
D193AR	GGCGGTGCGGTATTCGCTGAA	
D485AF	GTCGCCTTCATCTGGCGCTTC	This study
D485AR	GGCGACGAAGGCGCGGTGCAT	
N164AF	ACCGCCATCGAGGCGCTGAACTGG	This study
N164AR	CTCGATGGCGGTGTCCTGCCACTG	
R152EF	CGCCAGGAGCTGCGGGAAGACAGCG	This study
R152ER	CAGCTCCTGGCGGCCGAAGCGCAGG	

PalgPstIF	GCACTGCAGGCGGCCGCCTCCTCTTTCGG	This study
PalgHindIIIR	ACAAAGCTTGCATTCACCTCGATTGTTTG	This study

Table S2: Strains and vectors used in this study.

Strain or plasmid	Characteristics	Source
Strains		
P. aeruginosa		
PDO300	*mucA22* isogenic mutant derived from PAO1	(77)
PDO300Δ*algE*	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	(163)
Plasmids		
pPS865	Ap^r Gm^r; source of 1,100 bp *BamHI* fragment comprising *aacC1* gene flanked by Flp recombinase target site signal sequences	(74)
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(74)
pGEM®-T Easy	Ap^r, P_{lac}	Invitrogen
pGEM-TEasy:P*algD*	-879 bp relative *algD* ORF flanked by *PstI* and *HindIII* was ligated into pGEM-T Easy	This study
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	(93)
pBBR1MCS-5: *algE*	*HindIII-BamHI* fragment comprising *algE* inserted into vector pBBR1MCS-5	This study
pBBRMCS-5: *algE*M2FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after	This study

	the 225th bp of the ORF	
pBBRMCS-5: *algE*M4FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 381th bp of the ORF	This study
pBBRMCS-5: *algE*M6FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 513th bp of the ORF	This study
pBBRMCS-5: *algE*M8FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 627th bp of the ORF	This study
pBBRMCS-5: *algE*M10FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 756th bp of the ORF	This study
pBBRMCS-5: *algE*M12FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 939th bp of the ORF	This study
pBBRMCS-5: *algE*M14FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1149th bp of the ORF	This study
pBBRMCS-5: *algE*T2FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 408th bp of the ORF	This study
pBBRMCS-5: *algE*T4FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 651th bp of the ORF	This study
pBBRMCS-5: *algE*T6FLAG	algE fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 960th bp of the ORF	This study

pBBRMCS-5: *algE*T8-1FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1326th bp of the ORF	This study
pBBRMCS-5: *algE*T8-2FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1335th bp of the ORF	This study
pBBRMCS-5: *algE*T8-3FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 1365th bp of the ORF	This study
pBBRMCS-5: *algE*T5FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 795th bp of the ORF	This study
pBBRMCS-5: *algE*TrC9	*algE* fragment with a 27bp deletion at 5 end of the ORF	This study
pBBRMCS-5:*algE*K47E	*algE* fragment with three base pair substitution (Changing K into E) at position 47 a.a of mature protein	This study
pBBRMCS-5:*algE*R74E	*algE* fragment with three base pair substitution (Changing R into E) at position 74th a.a of mature protein	This study
pBBRMCS-5:*algE*R129E	a *algE* fragment with three base pair substitution (Changing R into E) at position 129th a.a of mature protein	This study
pBBRMCS-5:*algE*130A	*algE* fragment with three base pair substitution (Changing E into A) at position 130th a.a of mature protein	This study
pBBRMCS-5:*algE*R152E	*algE* fragment with three base pair substitution (Changing R into E) at position	This study

	152nd a.a of mature protein	
pBBRMCS-5:*algE*R154E	*algE* fragment with three base pair substitution (Changing R into E) at position 154th a.a of mature protein	This study
pBBRMCS-5:*algE*N164A	*algE* fragment with three base pair substitution (Changing N into A) at position 164th a.a of mature protein	This study
pBBRMCS-5:*algE*D162A	*algE* fragment with three base pair substitution (Changing D into A) at position 162nd a.a of mature protein	This study
pBBRMCS-5:*algE*E189A	*algE* fragment with three base pair substitution (Changing E into A) at position 189th a.a of mature protein	This study
pBBRMCS-5:*algE*D193A	*algE* fragment with three base pair substitution (Changing D into A) at position 193rd a.a of mature protein	This study
pBBRMCS-5:*algE*R353A	*algE* fragment with three base pair substitution (Changing R into A) at position 353rd a.a of mature protein	This study
pBBRMCS-5:*algE*R362A	*algE* fragment with three base pair substitution (Changing R into A) at position 362nd a.a of mature protein	This study
pBBRMCS-5:*algE*R364A	*algE* fragment with three base pair substitution (Changing R into A) at position 364th a.a of mature protein	This study
pBBRMCS-5:*algE*R365A	*algE* fragment with three base pair substitution (Changing R into A) at position 365th a.a of mature protein	This study

pBBRMCS-5:*algE*E368A	*algE* fragment with three base pair substitution (Changing E into A) at position 368th a.a of mature protein	This study
pBBRMCS-5:*algE*R459E	*algE* fragment with three base pair substitution (Changing R into E) at position 459th a.a of mature protein	This study
pBBRMCS-5:*algE*R461E	*algE* fragment with three base pair substitution (Changing R into E) at position 461th a.a of mature protein	This study
pBBRMCS-5:*algE*D485A	*algE* fragment with three base pair substitution (Changing D into A) at position 485th a.a of mature protein	This study
pBBRMCS-5: *algE*R353A+ R459E	*algE* fragment with three base pair substitutions Changing (R into A) at position 353rd a.a and (R into E) at 459th a.a of mature protein	This study
pBBRMCS-5: *algE*E368A+ R459E	*algE* fragment with three base pair substitutions Changing (E into A) at position 368th a.a and (R into E) at 459th a.a of mature protein	This study
pBBRMCS-5: *algE*R362A+ R459E	*algE* fragment with three base pair substitutions Changing (R into A) at position 362nd a.a and (R into E) at 459th a.a of mature protein	This study
pBBRMCS-5: *algE*D193A+ R362A	*algE* fragment with three base pair substitutions Changing (D into A) at position 193rd a.a and (R into E) at 362nd a.a of mature protein	This study
pBBRMCS-5:	*algE* fragment with three base pair	This study

*algE*R353A+ R459E+ K47E	substitutions Changing (R into A) at position 353rd a.a, (R into E) at 459th a.a and (K into E) at 47th a.a of mature protein	
pBBRMCS-5: *algE*R353A+ R459E+ R74E	*algE* fragment with three base pair substitutions Changing (R into A) at position 353rd a.a, (R into E) at 459th a.a and (R into E) at 74th a.a of mature protein	This study
pBBRMCS-5: *algE*R362A+ R459E+ K47E	*algE* fragment with three base pair substitutions Changing (R into A) at position 362nd a.a, (R into E) at 459th a.a and (K into E) at 47th a.a of mature protein	This study
pBBRMCS-5: *algE*R362A+ R459E+ R74E	*algE* fragment with three base pair substitutions Changing (R into A) at position 362nd a.a, (R into E) at 459th a.a and (K into E) at 74th a.a of mature protein	This study
pBBRMCS-5: *algE*E368A+ R459E+ K47E	*algE* fragment with three base pair substitutions Changing (E into A) at position 368th a.a, (R into E) at 459th a.a and (K into E) at 47th a.a of mature protein	This study
pBBRMCS-5: *algE*E368A+ R459E+ R74E	*algE* fragment with three base pair substitutions Changing (E into A) at position 368th a.a, (R into E) at 459th a.a and (R into E) at 74th a.a of mature protein	This study
mini-CTX-*lacZ*	Integration proficient vector	(75)
miniCTX:P*algE*	*algE* frgment with *algD* promoter cloned into chromosomal integration vector	This study
miniCTX:P*algE*T 2F	*algE* fragment with a 24 bp insertion inserted after the 408th bp of the ORF with *algD* promoter cloned into the chromosomal	This study

	integration vector	
miniCTX:P*algE*T 4F	*algE* fragment with a 24 bp insertion, inserted after the 651th bp of the ORF with *algD* promoter cloned into the chromosomal integration vector	This study
miniCTX:P*algE*T 5F	*algE* fragment with a 24 bp insertion, inserted after the 795th bp of the ORF with *algD* promoter cloned into the chromosomal integration vector	This study
miniCTX:P*algE*T 6F	*algE* fragment with a 24 bp insertion, inserted after the 960th bp of the ORF with *algD* promoter cloned into the chromosomal integration vector	This study

Chapter V

Insight into assembly of the alginate biosynthesis machinery in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen of particular significance to cystic fibrosis patients. This bacterium produces the exopolysaccharide alginate, which confers resistance to antibiotics and leads to poor prognosis for patients.

The proteins required for alginate polymerisation and secretion are encoded by genes organized in a single operon. It has been proposed that these proteins form a multiprotein complex which spans from the inner to outer membrane. Here experimental evidence was provided supporting such a multiprotein complex through mutual stability analysis, pull-down assays and co-immunoprecipitation. The impact of the absence of single proteins, subunits, on this multiprotein complex i.e. the stability of potentially interacting proteins as well as on alginate production was investigated. Deletion of *algK* in an alginate-overproducing strain, PDO300, interfered with the polymerisation of alginate suggesting that in absence of AlgK, the polymerase and co-polymerase subunits, Alg8 and Alg44, are destabilised. Based on mutual stability analysis interactions between AlgE (outer membrane)-AlgK (periplasm)-AlgX (periplasm)-Alg44 (inner membrane)-Alg8 (inner membrane)-AlgG (periplasm) were proposed. Co-immunoprecipitation using a FLAG tagged variant of outer membrane protein, AlgE, demonstrated an interaction with the periplasmic protein AlgK. Pull-down assays using histidine tagged AlgK showed that AlgK interacts with another periplasmic protein AlgX. AlgX in turn was also co-purified with histidine tagged Alg44 located to the inner membrane. Detection of AlgG and AlgE in PAO1 supported the existence of internal promoters controlling expression of the respective genes. Overall experimental evidence was provided for the existence of a multiprotein complex required for alginate polymerisation and secretion.

Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen responsible for many nosocomial infections. Upon infection of the lungs of cystic fibrosis (CF) patients, it converts to a mucoid phenotype, characterised by the overproduction of the exopolysaccharide alginate which is a polymer of β-1, 4 linked β-D mannuronic acid and its C-5 epimer α-L guluronic acid. Alginate not only protects the pathogen from antibiotics and host immune responses, but also clogs the patient's lungs; thus the prognosis for patients is poor. These infections are notoriously difficult to eradicate and often lead to the death of the patient (72, 130).

Twelve proteins required for the biosynthesis of alginate are encoded by the *alg* operon (*algD*/*8*/*44*/*K*/*E*/*G*/*X*/*L*/*I*/*J*/*F*/*A*) while another protein, AlgC, is encoded elsewhere (23, 48). The cytoplasmic proteins AlgA, AlgC and AlgD convert D-fructose-6-phosphate through a series of reactions into GDP-mannuronic acid, the activated precursor for alginate biosynthesis (107, 144, 197). GDP-mannuronic acid is polymerised into a polymannuronate chain by the inner membrane glycosyltransferase, Alg8, with some involvement by Alg44 (108, 120, 138). Alg8 has multiple transmembrane domains and a large cytoplasmic glycosyltransferase domain (120) while Alg44 has a single transmembrane domain separating a cytoplasmic c-di-GMP binding PilZ domain and a large C-terminal periplasmic domain (108). Upon polymerisation, the nascent polymannuronate chain is proposed to be translocated across the periplasm by a periplasmic scaffold composed of AlgG, AlgL, AlgK, AlgX and AlgE (69, 80-82, 143). In the periplasm, mannuronic acid residues of the nascent alginate chain are selectively O-acetylated at O2' and/or O3' positions by the action of AlgI, AlgJ and AlgF (50, 51). A component of the proposed periplasmic scaffold, AlgG, is an epimerase which converts D-mannuronic acid into α-L guluronic acids at polymer level (46, 80). AlgL, a bi-functional periplasmic protein, serves as an alginate degrading enzyme while also contributing to the integrity of the periplasmic scaffold (3, 82). AlgK is an outer membrane lipoprotein located to the periplasm and it contains multiple copies of tetratricopeptide-repeat motifs, a widespread domain involved in protein-protein interactions (86). The protein AlgX is also secreted to the periplasm and interacts with AlgK and MucD, a serine protease which is involved in the post-translational regulation of alginate biosynthesis (60, 71) . The exact functions of AlgK and AlgX are not clear; however, they are essential for alginate secretion (81, 110, 143). The nascent alginate is

secreted out of the cell through AlgE, which forms an electropositive β-barrel pore in the outer membrane (69, 131, 184).

Recent studies have suggested that proteins involved in the biosynthesis of alginate form a multiprotein complex which spans the entire bacterial envelope fraction (140). In the current study, the role of individual proteins (Alg8, Alg44, AlgE, AlgX, AlgK and MucD) on the stability of other proposed subunits and the whole alginate biosynthesis complex was investigated. In order to apply mutual stability isogenic knock-out mutants (PDO300Δ*algX*, PDO300Δ*algK*, PDO300Δ*alg44*, PDO300Δ*algE*, PDO300Δ*alg8* and PAO1Δ*mucD*) of genes encoding proposed subunits were generated. These mutants were complemented *in cis* i.e. the affected gene was integrated into the genome to avoid copy number effects on mutual stability. Furthermore co-immunoprecipitation (Co-IP) and pull-down assays were performed, using FLAG tagged variant of AlgE and hexahistidine tagged Alg44 and AlgK, to identify interactions between various components.

Materials and Methods

Construction of isogenic knock-out mutants.

PDO300Δ*algK* was generated as follows. Fragments from N-terminus (1-460bp) and C-terminus (1045-1428bp) of the coding region of *algK* gene were amplified using *Taq* polymerase and primer pairs algKNEcoF and algKNBamR, and algKCBamF and algKCEcoR, respectively. The PCR products were hydrolysed using *BamHI* and ligated together into pGEM®-T Easy vector (Promega, Madison, USA) yielding pGEM-TEasy:*algK*NC. A 1,100 bp fragment, FRT-*aacC1*-FRT, encoding a gentamycin resistance gene (*aacC1*) flanked by FLP-recombinase recognition sites (FRT), was obtained by digesting the plasmid pPS856 with *BamHI* (74). The resulting fragment FRT-*aacC1*-FRT was inserted into pGEM-TEasy:*algK*NC, yielding the construct pGEM-TEasy:*algK*NCΩGm. The plasmid pGEM-TEasy:*algK*NCΩGm was used as a template for amplifying the *algK*NCΩGm cassette with *Pfx* high fidelity polymerase using the primers algKNEcoF and algKCEcoR. The subsequent *algK*-gentamycin-resistance cassette was ligated into the *SmaI* site of the suicide vector pEX100T, yielding pEX100T:Δ*algK*ΩGm. This plasmid was transferred into *P. aeruginosa* PDO300 via conjugation utilising the *E. coli* S17-1 (53, 163). Transconjugants were selected for on Mineral Salt Medium (MSM) supplemented with 100 μg/mL of gentamycin and 5% (w/v) sucrose (153). The double crossover event, generating the strain PDO300Δ*algK*ΩGm, was confirmed by gentamycin resistance (300 ug/ml) and PCR by using the primers algK(upXout) and algK(downXout). *E coli* SM10 was used as the donor strain to transfer the Flp recombinase encoding vector pFLP2 (74) into PDO300Δ*algK*NCΩGm to remove the gentamycin resistance cassette (FRT-*aacC1*-FRT). Successful transconjugants were selected on Pseudomonas Isolation Agar (PIA) containing carbenicillin (300 ug/ml) and subsequently transferred to PIA plates containing 5% sucrose to remove the pFLP2 plasmid. Successful loss of the FRT-*aacC1*-FRT cassette was confirmed by PCR and sensitivity to gentamycin and carbenicillin. Plasmids pEX100T:Δ*algE*ΩGm and pEX100T:Δ*algX*ΩGm was used to knock-out *algE* in PAO1 (generating PAO1Δ*algE*) and *algX* in PDO300Δ*alg44* (generating PDO300Δ*alg44*Δ*algX*) using the same strategy as described above. The bacterial strains and sequence of primers used for PCR is given in Table S1 and S2.

***In trans* complementation of knock-out mutants.**

Plasmids pBBR1MCS-5:*algK* and pBBR1MCS-5:*algK*-6*his* were generated as follows. The *algK* gene and a C-terminal hexahistadine translational fusion were amplified using primer pairs algKN(HiSDNd) and algKC(Ba), and algKN(HiSDNd) and algKC(6xHBa). These PCR products were ligated into pGEM®-T Easy producing the plasmids pGEM-TEasy:*algK*(HiSNd-Ba) and pGEM-TEasy:AlgK(HiSNd-6xHBa). These plasmids were digested with *BamHI* and *HindIII*, releasing the fragments *algK*(HiSDNd-Ba) and *algK*(HiSNd-6xHBa), which were independently ligated into the corresponding sites of pBBR1MCS-5 (93) yielding the final constructs pBBR1MCS-5::*algK* and pBBR1MCS-5:*algK*-6*his* Resulting plasmids were electroporated into *P. aeruginosa* PDO300Δ*algK* and transformants selected on PIA agar containing 300 μg/mL of gentamycin.

The plasmid pBBR1MCS-5:*algE* (69) was used to complement the PAO1Δ*algE* mutant. The double mutant PDO300Δ*alg44*Δ*algX* was complemented by plasmid pBBR1MCS-5:*alg44algX*. The oligonucleotides sequences are given in Table S2.

***In cis* complementation by chromosomal integration of respective genes.**

To generate miniCTX:P*mucD* the promoter region -901 bp relative to *algU* open reading frame was amplified using primers palgUHindIIIF and palgUXbaIR (156) and hydrolysed with *HindIII* and *XbaI*, the *mucD* region was hydrolysed form pBBR1MCS-5:*mucD* (71) using *Xba*I and *Sac*I and purified and ligated together with *algU* promoter in mini-CTX-*lacZ* (158) hydrolysed with *Hind*III and *Sac*I generating miniCTX:P*mucD*. To generate plasmids miniCTX:P*alg44,* miniCTX:P*alg8,* miniCTX:P*algK,* miniCTX:P*algX,* and miniCTX:P*algE* the *algD* promoter fragment was hydrolysed from pGEM-TEasy:P*algD* with *Pst*I and *Hind*III and ligated together with, *HindIII* and *BamHI* hydrolysed *alg44*, *alg8*, *algK*, *algX* and *algE*, into mini-CTX-*lacZ* hydrolysed with *PstI* and *BamHI*.

These plasmids (miniCTX:P*alg44,* miniCTX:P*alg8,* miniCTX:P*algX,* miniCTX:P*algE,* miniCTX:P*mucD and* miniCTX:P*algK*) were electroporated into PDO300Δ*alg44*, PDO300Δ*alg8*, PDO300Δ*algX*, PDO300Δ*algE*, PAO1Δ*mucD* described in previous studies (61, 69, 71, 138, 140) as well as into PDO300Δ*algK*, respectively. Transformants were selected for on PIA supplemented with 150 μg/ml tetracycline. The chromosomal integration of the plasmids was confirmed through PCR using primers

PserUP and PserDOWN. The mini-CTX-*lacZ* backbone was removed by introducing the Flippase encoding pFLP2 plasmid. The pFLP2 plasmid was cured by cultivating the cells for 24 h on PIA containing 5% sucrose. Cells sensitive to tetracycline and carbenicillin were analysed through PCR for removal of mini-CTX-*lacZ* backbone. The same strategy was used to integrate the empty vector mini-CTX-*lacZ* into the chromosome of mutant strains as well as PDO300 and PAO1. The oligonucleotide sequence can be found in the table S2.

Isolation of envelope fraction.

Cells of *P. aeruginosa* were grown overnight in LB media in planktonic mode or for 48 h on PIA on solid media. Cells were harvested by centrifugation or scrapping from the plates and washed twice in 100 ml of 10 mM HEPES pH 7.4 buffer, containing Roche Complete Mini EDTA-free protease inhibitor. Cells were resuspended in 10 ml of 10 mM HEPES buffer and lysed by sonication on ice for 12 cycles with 15 s of sonication followed by 15 s of cool down. After lysis cells were centrifuged at 8000*g* for 45 min at 4°C to remove the unbroken cells and cellular debris. The soluble fraction containing the whole cell lysate was centrifuged at 100,000*g* for 1 h at 4 °C to isolate the envelope fraction (inner membrane, outer membrane and associated proteins). Envelope fractions were used immediately or stored at -80°C for future analysis.

Co-immunoprecipitation of AlgE and pull-down assays.

To solubilise the envelope fractions they were suspended in buffer A (100 mM $NaH_2PO_4xH_20$, 10 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM KCl and 1% n-octyl-β-D-glucopyranoside) by gently rocking at 4°C for 2 h and insoluble material removed by centrifugation at 50,000*g* for 30 min.

For FLAG tagged AlgE (AlgEL6F), 1 ml of supernatant was incubated with 40 µL of anti-FLAG IP resin slurry (GenScript, Piscataway, NJ) overnight at 4°C. The mixture was centrifuged at 5000*g* for 30 s and supernatant was removed. Resin was washed three times with equilibration buffer (50 mM Tris, 150 mM NaCl, pH 7.4). 20 ul of 1X SDS-PAGE loading buffer (62.5mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 0.01% w/v bromophenol blue) was added to the resin and heated at 100°C for 5 min. Resin was again centrifuged at 8000*g* for 30 s and supernatant was subjected to SDS-PAGE and analysed by and western blotting.

For hexahistidine tagged AlgK (AlgK-His) pull-down, the His-Spin Protein Miniprep™ kit (Zymo Research, USA) was used according to manufacturer's instructions, with a few modifications. The solubilised envelope fraction (in buffer A) was incubated with the resin. The affinity gel was washed twice, once with each wash buffer (supplemented with imidazole at 25 and 50 mM, respectively). Proteins of interest were eluted from the affinity gel by incubation for 1 min with 100 µl elution buffer (containing 500 mM imidazole) and centrifugation. The eluted fraction was either stored at -80°C or analysed by SDS-PAGE and immunoblotting.

***In vivo* chemical crosslinking.**

Chemical crosslinking was performed using disuccinimidyl glutarate (DSG, (Pierce Biotechnology, Rockford, IL)) prepared in DMSO. Briefly, 400 ml overnight culture of *P. aeruginosa* in LB were harvested and washed twice with saline solution and once with PBS (pH 7.5). 5 ml of cells were incubated with DSG (1.5 mM) for 30 min at 37°C or 2 h on ice. Reactions were terminated by adding Tris-HCl (pH 7.5) at final concentration of 20 mM and incubating at room temperature for 15 min. Cells were pelleted and resuspended in 5 ml of TBS (pH 7.8) and treated with 0.5 ml of lysis buffer (150 mM NaCl, 100 mM Tris-HCl, 0.2% Triton X-100, pH 8.0) with 1 mg/ml lysozyme, 1mg/ml *DNase* and protease inhibitor cocktail set III, free-EDTA (Calbiochem Co.). Samples were incubated on ice for 20 min with shaking and subsequently lysed by sonication. Cell debris was removed by centrifugation at 15,800*g* for 30 min at 4°C and supernatants were subjected to centrifugation at 100,000*g* for 1 h. The pellet constituting the envelope fraction was solubilised in solubilising buffer (8M Urea, 100 mM Tris-HCl (pH 8.0), 1 M NaCl, 1% Triton X-100, 0.2% *N*-laurol sarcosine, 10 mM Imidazole). Histidine tagged Alg44 (Alg44-His) was purified using His-Spin Protein Miniprep™ (Zymo Research, USA) as described above. Purified proteins were separated by 4–15% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) SDS-PAGE and identified through immunoblotting.

Protein analysis.

A total of 20 µg of protein was loaded and separated by SDS-PAGE using 8% polyacrylamide gels. Resulting gels were either stained by Coomassie Blue or used for immunoblotting using iBlot® dry blotting system (Invitrogen). Nitrocellulose membranes were blocked using skim milk (5% w/v) in Tris-buffered-saline containing

Tween-20 (0.05% v/v) (TBST) for 1 h at room temperature or overnight at 4°C. After washing the membrane three times with TBST, it was treated with the primary antibody, raised in rabbit, of interest: anti-Alg44 (1:10,000), anti-AlgK(1:10,000), anti-AlgG(1:1000), anti-AlgX(1:7000) and anti-AlgE (1:5000) in TBST containing skim milk (2% w/v) for 1 h at room temperature. Membranes were again washed three times and incubated with the commercial secondary antibody (Abcam, Cambridge, UK), anti-IgG anti-rabbit antibodies labelled with HRP (1:10,000) in TBST for 1 h at room temperature. After three washes, membranes were resolved with SuperSignal® West Pico chemiluminescent substrate (Thermoscientific, Rockford, IL) and developed on X-ray film (KODAK, Rochester, New York). For the detection of hexahistadine tagged proteins the HisProbe™-HRP kit (Thermoscientific, Rockford, IL) was used according to the manufactures instructions.

Alginate quantification.

Alginate was isolated and uronic acid content quantifid as described previously (69). To assess the free uronic acids 2 ml of overnight culture was centrifuged and supernatant filtered through vivaspin-500 (GE Healthcare) filter device with molecular weight cut off of 10 kDa. The uronic acids in the flow through, which consist of free uronic acids and short length alginate degradation products was measured.

Solid surface attachment assay.

Attachment of cells to solid surface was measured as has been described (70). Briefly, relevant strains of *P. aeruginosa* were grown overnight and OD 600 nm was measured. Using an appropriate amount of culture a final dilution of 1:100 in LB media was made. Eight wells of three sterile microplates (Greiner bio-one, USA) were inoculated with 100 µl of diluted culture and incubated at 37°C for 2 h, 4 h, 6 h and 24 h. After incubation non-adherent bacteria were removed by gentle washing procedure as has been described (70).

Results

Effect of *algK* deletion on alginate biosynthesis.

A marker free isogenic knock-out mutant of *algK* was generated in *P. aeruginosa* PDO300 to investigate its role in alginate biosynthesis. Immunoblot analysis of envelope fractions isolated from PDO300Δ*algK* showed that AlgK was absent (Fig. 1A). Similar to what has been described previously (81), this strain did not produce detectable levels of alginate (Fig. 1B).

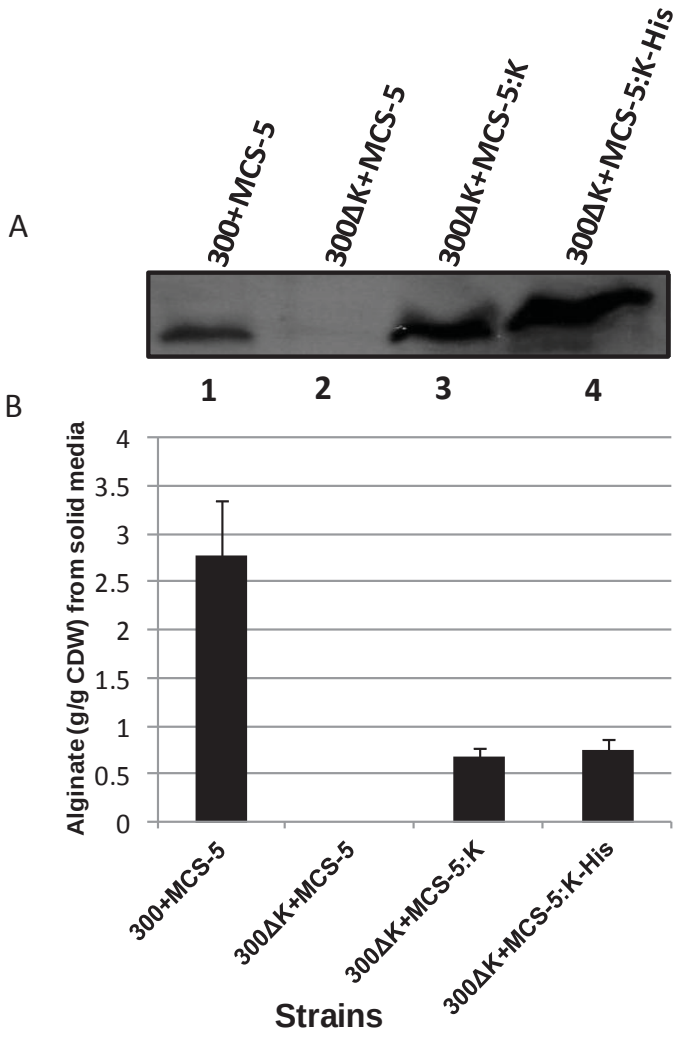


Fig. 1: Complementation of the *algK* knock-out mutant. (A) Immunoblot analysis was performed to test the presence of AlgK in envelope fractions isolated from cells grown on solid media. AlgK was detected in wildtype, alginate producing, strain PDO300+pBBR1MCS-5 (Lane 1, A and B). However, no alginate or AlgK was detected in the *algK* knock-out mutant PDO300Δ*algK*+pBBR1MCS-5 (Lane 2, A and B). The presence of AlgK and alginate production was restored in *algK* (with or without a histidine tag) complemented strains, PDO300Δ*algK*+pBBR1MCS-5:*algK* and

PDO300Δ*algK*+pBBR1MCS-5:*algK-6his* (Lane 3 and 4 respectively, A and B). (B) The amount of alginate produced by strains grown on solid media is given as grams of alginate per gram of cellular dry weight. All the experiments were conducted in triplicates and error bar represents the standard deviation from the mean value. ENV: Envelope; IB: Immunoblotting; CDW: Cellular Dry Weight; SD: Standard deviation.

Recent studies have shown that deletion of AlgG, AlgX and AlgE resulted in the secretion of free uronic acids, which are considered as degradation products of alginate, caused by the action of alginate lyase, AlgL (69, 80, 143). To assess whether the loss of mucoidity of the *algK* mutants is caused by the inability to polymerise alginate or because of its degradation in the periplasm, uronic acid quantification was performed. Contrary to what has been described previously no free uronic acids were detected for *algK* deletion mutant (Fig. 2). Similar to *algK* mutant no free uronic acids were produced by by *alg44* and *algX* mutants of PDO300 (Fig. 2). These results suggested that deletion of *algK* has an effect on the stability of Alg44 and/or Alg8 leading to a defect in the polymerisation of alginate.

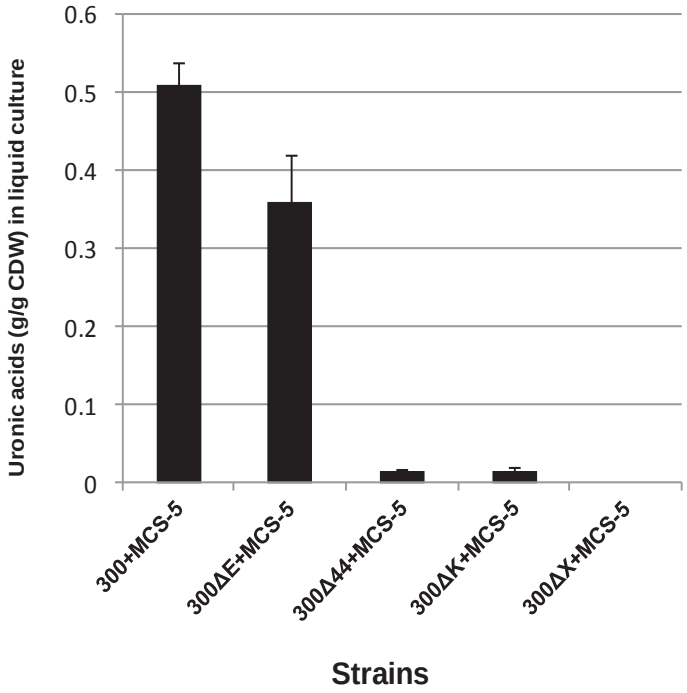


Fig. 2: Amount of free uronic acids produced by various strains when grown in liquid culture. Cells grown overnight in liquid media were sedimented and free uronic acids were measured in the cell-free supernatant. Uronic acids were detected for the

wild type, 300+MCS-5 (PDO300(pBBR1MCS-5)) and *algE* knock-out strains, 300ΔE+MCS-5 (PDO300Δ*algE*(pBBR1MCS-5)). However, almost no uronic acids were detected for knock-out mutant of *alg44*, [300Δ44+MCS-5, (PDO300Δ*alg44*(pBBR1MCS-5))], *algK*, [300ΔK+MCS-5, (PDO300Δ*alg44*(pBBR1MCS-5))], and *algX*, [300ΔX+MCS-5, (PDO300Δ*algX*(pBBR1MCS-5))]. Amount of uronic acids (g/g of CDW) is given as mean of triplicates with error bar representing standard deviation. CDW: Cellular Dry Weight; SD: Standard deviation.

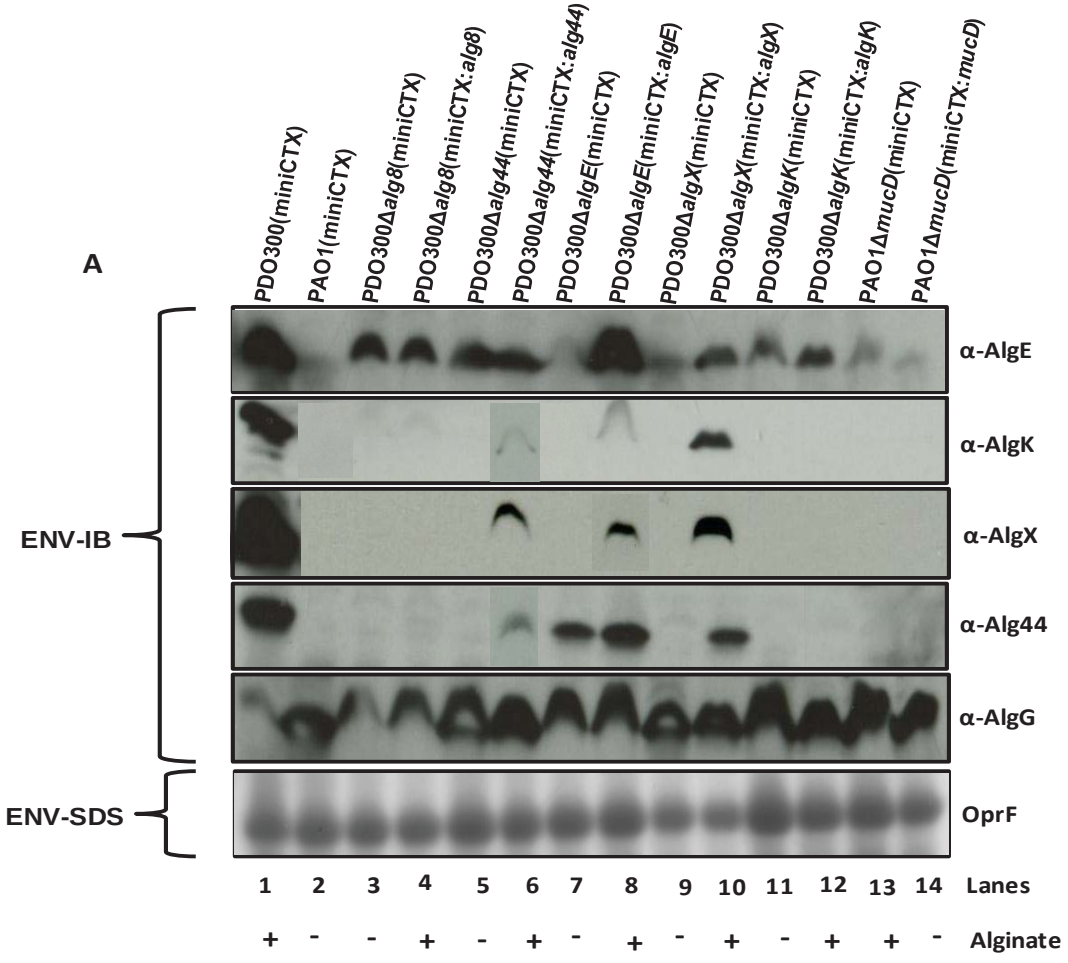
Complementation of the *algK* knock-out mutant.

In order to rule out the possibility of polar effects resulting from the deletion of *algK*, PDO300Δ*algK* was complemented with *algK* either *in trans*, pBBR1MCS-5:*algK*, or *in cis*, miniCTX:*algK*. The presence of AlgK was restored in the complemented strains (Fig. 1A, S1 lane 14). Interestingly, AlgK was only detected in the envelope fractions when cells were harvested from solid media but was absent when envelopes were obtained from cells grown in liquid media (Fig. 3 lane 12, S1 lane 14). The amount of alginate produced by PDO300Δ*algK*(pBBR1MCS-5:*algK*) was 4-fold less than PDO300(pBBR1MCS-5) (Fig. 1B) and the alginate produced by PDO300Δ*algK*(miniCTX:*algK*) was 4.4 fold less than the PDO300(miniCTX) (Fig. 3B). AlgK with a hexahistidine tag (AlgK-His) also restored alginate production and the presence of the AlgK protein in the envelope fraction (Fig. 1A, 1B).

Mutual stability analysis.

Recent studies have suggested the existence of a multiprotein complex involved in the polymerisation and secretion of alginate (140). Here the stability of the complex was assessed by removing individual subunits of the proposed envelope-spanning multiprotein complex. The impact of proposed subunits on the stability of potentially interacting subunits was investigated. Mutants of PDO300 lacking *algE*, *algX*, *algK*, *alg8*, *alg44* and PAO1 lacking *mucD* were used. These mutants were complemented *in cis* to address stoichiometric issues; here, *in cis* complementation introduces a single copy of a gene under the control of its native promoter into the chromosomal DNA of the respective strain. Strains PDO300 and PAO1 were used as positive and negative controls, respectively. The proteins AlgE, AlgX, AlgK, AlgG and Alg44 were detected in the envelope fraction of PDO300 (Fig. 3A lane 1). As expected, AlgX and Alg44 were absent in the envelope fractions of PAO1, which does not produce detectable

amount of alginate as shown by uronic acid analysis. Interestingly, a faint band for AlgE and a distinct band for AlgG were detected in PAO1 (Fig. 3A lane 2, S1 lane 2). Deletion of *alg8* resulted in the absence of AlgK, AlgX and Alg44 from the PDO300Δ*alg8* envelope fraction (Fig. 3A lane 3). Expression of *alg8 in cis* could not restore these proteins to the detectable levels (Fig. 3A lane 4). The presence and absence of Alg8 did not appear to affect the stability of AlgE (Fig. 3A lane 3 & 4). AlgG was detected in both the Δ*alg8* mutant and its complemented strain PDO300Δ*alg8*(miniCTX:*alg8*) at a level higher than the wild type PDO300 (Fig. 3A lane 3 & 4), though AlgG was present in slightly higher amounts in the complemented mutant.



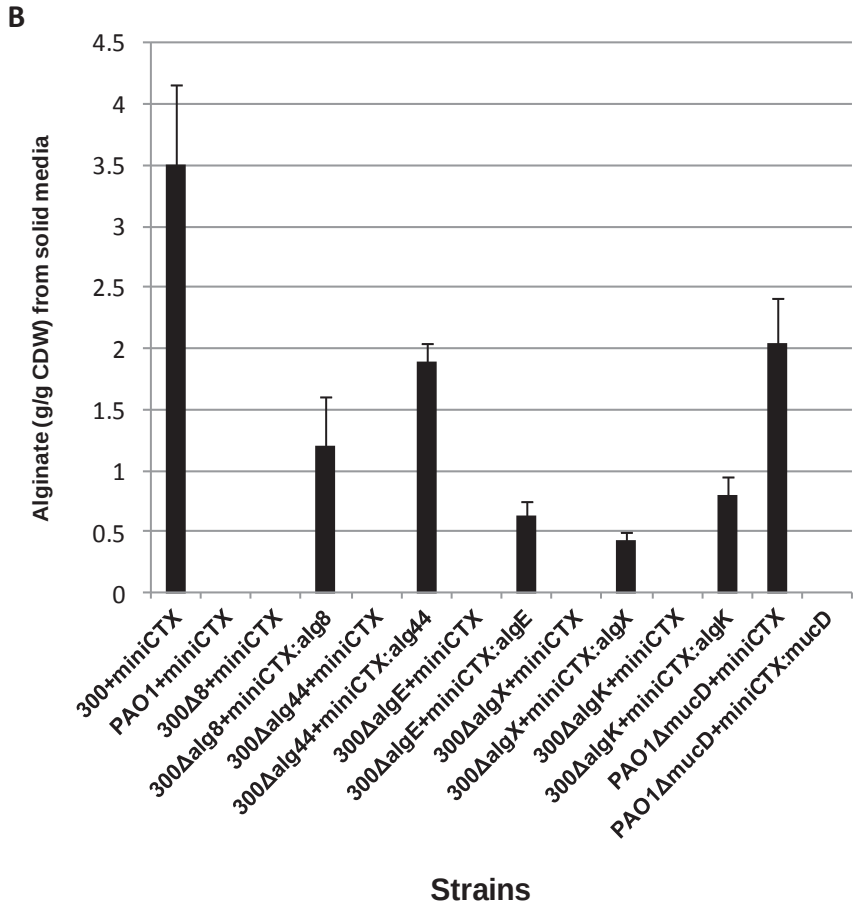


Fig. 3: Effect of the absence or presence of proposed subunits of the alginate biosynthesis machinery on the stability other subunits in the multiprotein complex and alginate production. (A) Envelope fractions from PDO300, PAO1, knock-out mutants of *alg8*, *alg44*, *algE*, *algX*, *algK* and *mucD* and their *in cis* complemented strains, were isolated after overnight growth in planktonic mode and subjected to immunoblot analysis. The envelope fractions were probed for the presence or absence of various components of alginate polymerisation and secretion complex using antibodies as indicated (Lane 1-14).Some parts of this image are copied from other blots, which showed a clear band for respective protein. All these WB experiments were performed at least two times ENV: Envelope fraction, SDS-ENV: SDS-PAGE of envelope fraction. Only relevant parts of the blots were shown. (+) and (–) sign for each strain indicates that alginate was either detected or not, respectively. Constitutively expressed OM protein OprF was used as loading control. (B) The amount of alginate (g/g of CDW) produced by each strain was assessed from solid media. The amount of alginate given here is the mean of triplicates with error bar representing the standard deviation. CDW: Cellular Dry Weight; SD: Standard deviation.

Deletion of *alg44* destabilised AlgK and AlgX but not AlgE and AlgG (Fig. 3A lane 5). Complementation with *alg44* restored AlgK and AlgX (Fig. 3A lane 6). These findings indicate that Alg44 potentially interacts with either AlgX or AlgK or both.

Deletion of *algE* resulted in AlgK and AlgX not being detected while levels of Alg44 were reduced (Fig. 3A lane 7). Expression of *algE in cis* restored the presence of AlgK, AlgX and restored the levels of Alg44 (Fig. 3A lane 8). These results are consistent with the proposed interaction between AlgE and AlgK which in turn interacts with AlgX (71, 86).

Interestingly, deletion of *algX* (PDO300Δ*algX*) destabilised all of the components (AlgE, AlgK, Alg44) except AlgG (Fig. 3A lane 9). All of the components were restored when *algX* was expressed *in cis* (Fig. 3A lane 10). AlgX has been shown to interact with AlgK (71). The destabilisation of AlgK in *algX* mutant further supports this interaction. The destabilization of AlgE observed in the *algX* mutant might be an indirect effect of the destabilization of AlgK. However, deletion of AlgK alone did not completely destabilize AlgE. These results indicated that AlgX plays a role in localisation of AlgE to the OM.

In the strain PDO300Δ*algK,* AlgX and Alg44 were completely destabilized while levels of AlgE were reduced (Fig. 3A lane 11). Interestingly, the presence of these components could not be restored in the *algK* complemented mutants during planktonic growth (Fig. 3A lane 12). The *algK in cis* complemented strains did not produce AlgK at detectable levels. Though, intriguingly when cells were harvested from solid media (as opposed to growth in liquid culture) AlgK was restored to normal levels (Fig. S1, lane 14).

Unexpectedly, a mutant of PAO1 lacking *mucD,* a negative regulator of alginate biosynthesis, did not show detectable levels of Alg44, AlgK and AlgX when cells were grown in planktonic mode (Fig. 3A lane 13), however, in PAO1Δ*mucD* AlgE, AlgK, AlgX, Alg44 and AlgG were detected when cells were grown on solid media and alginate was produced (Fig. 3B, Figure S1 lane 11).

Deletion of *alg8*, *algE*, *algK*, *algX* and *alg44* in PDO300 caused a complete loss in the production of alginate. Production of alginate was restored, although the amount of alginate was less than the wild type strain PDO300, when these components were expressed *in cis* in their respective knock-out strains (Fig. 3B). Among all the strains

algK, *algX* and *algE* complemented strains produced the lowest amount of alginate. This suggests a strict stoichiometric requirement of these proteins for the formation of functional alginate biosynthesis complexes.

Complementation of PDO300Δ*alg8* with *alg8* or PDO300Δ*algK* with *algK*, respectively, restored the presence of proposed subunits of the alginate biosynthesis complex when cells were grown on solid media, which ruled out any polar effects caused by these deletions (Fig. S1, lane 4 & 14). Alginate is overproduced during the biofilm mode of growth (73) which suggested that on solid media the transcription from *algD* operon increases and/or the alginate biosynthesis multiprotein complex is more active/abundant.

AlgK interacts with AlgE and AlgX *in vivo*.

Our mutual stability analysis suggested an interaction between AlgE and AlgK. This interaction has been previously proposed, based on structural domain homologies to other proteins (86). AlgK contain tetratricopeptide-repeats (TRP) which are involved in protein-protein interaction. Furthermore it has been proposed that, BcsC and PgaA proteins involved in the secretion of cellulose and *N*-acetyl-D-glucosamine, respectively, have a domain architecture which represents fusion of AlgK and AlgE (79, 185). Despite these precedences no direct evidence had been provided for this long proposed interaction between AlgE and AlgK (86, 184). To investigate this further, a co-IP assay was performed by expressing in PDO300Δ*algE,* a variant of AlgE with the FLAG epitope inserted into the extracellular loop 6 (pBBR1MCS-5: *algEL6F*). Wild type AlgE with no FLAG epitope was used as a negative control. Anti-FLAG IP resin was used to isolate AlgEL6F from solubilised envelope fractions of cells harvested from solid media. A distinct band of AlgE and AlgK was detected in the elution fraction. As expected, no band for AlgE or AlgK was detected in the negative control (Fig. 4A). The elution fraction was also probed with anti-AlgX and anti-Alg44 antibodies but no band for AlgX or Alg44 was detected.

To investigate the proposed AlgK-AlgE interaction from the perspective of AlgK, a pull-down assay using AlgK-His (AlgK with a C terminal hexahistidine tag) were done. To this end AlgK-His and wild type AlgK (negative control) were independently

expressed in PDO300Δ*algK in trans*. Cells were either grown in liquid media and treated with cross-linkers or were harvested directly from solid media without cross-linking. AlgK-His was purified form solubilised envelope fractions of these cells and analysed by immunoblotting. However, under these experimental conditions AlgE was not co-purified with AlgK-His. To determine whether AlgK interacts with other components, immunoblotting with AlgX and Alg44 antibodies was carried out. We found only AlgX was co-purified with AlgK-His (Fig. 4B). As expected, AlgK and AlgX were not detected in the negative control (Fig. 4B).

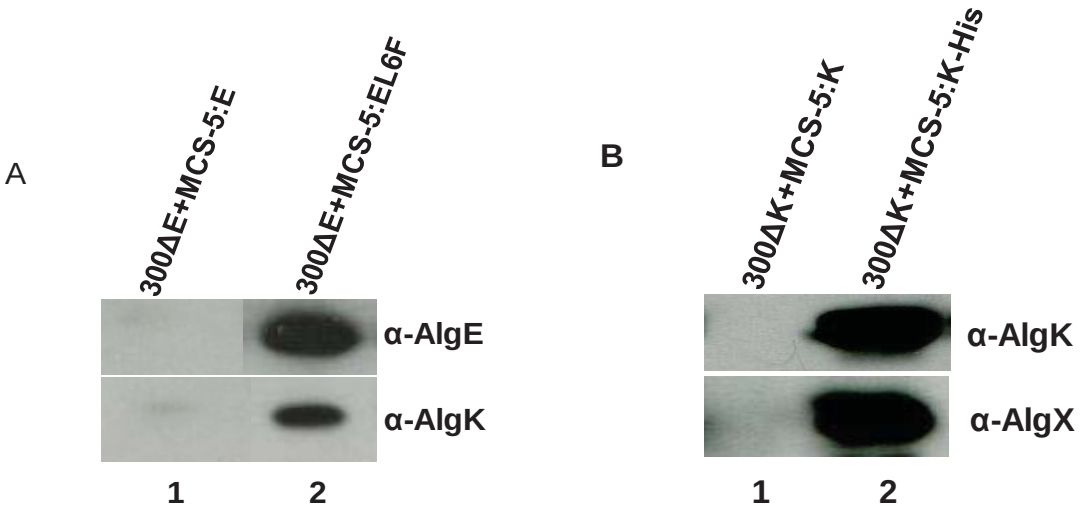


Fig. 4: Co-IP and pull-down assays show AlgK interacts with AlgE and AlgX. (A) Solubilised envelope fractions from cells, grown on solid media. of *P. aeruginosa* PDO300Δ*algE* carrying either plasmid (pBBR1MCS-5:*algE*) (Lane 1) or (pBBR1MCS-5:*algEL6F*) (lane 2) were incubated with anti-FLAG co-IP resin. The elution fraction was probed with specific antibodies as previously mentioned. (B) A Pull-down assay using AlgK-His was performed. *P. aeruginosa* PDO300Δ*algK* harbouring either (pBBR1MCS-5:*algK*) (Lane 1) or (pBBR1MCS-5:*algK-6his*) (Lane 2) were grown on solid media and envelope fraction were isolated. Solubilised envelope fractions were subjected to metal ion affinity chromatography and probed with antibodies as indicated.

Interaction between Alg44 and AlgX.

Results from mutual stability experiments suggested an interaction between Alg44 and AlgK/AlgX. To investigate this further, we carried out a pull-down assay using PDO300Δ*alg44* (138) carrying plasmids for expression of either wild type Alg44 or Alg44-His (Alg44 with hexahistidine tag). Wild type Alg44 was used as a control. A non-cleavable chemical cross-linker, DSG, with spacer arm length of 7.7Å for stabilisation of any protein-protein interactions was used. As expected, these pull-down experiments using immobilised metal ion affinity chromatography and the control strain PDO300*Δalg44*(pBBR1MCS-5:*alg44*) did not enable purification Alg44 (Fig. 5). However, with cell lysates containing Alg44-His, an additional protein with an apparent molecular weight of ~90 kDa was detected which bound both anti-Alg44 and anti-AlgX antibodies (Fig. 5A, 5B). The molecular weight of this protein is approximately equal to the combined molecular weight of Alg44 and AlgX (91.57 kDa). However, in the absence of cross-linker or purifying Alg44-His from a mutant deficient in *alg44* and *algX* (PDO300Δ*alg44*Δ*algX*) this ~90 kDa was not any more detectable (Fig. 5A, 5B). These results suggested that *in vivo* Alg44 interacts with AlgX. Under the experimental conditions used here neither AlgK nor AlgE did co-purify with Alg44.

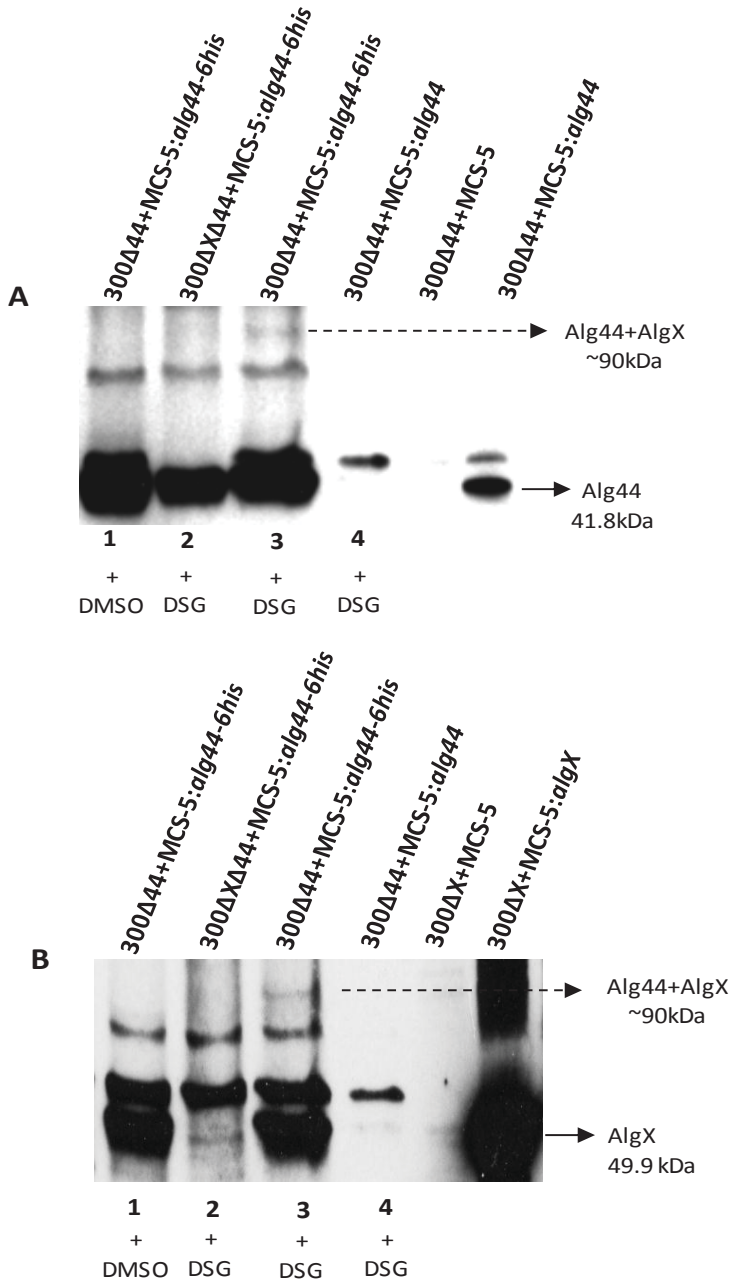


Fig. 5: *In vivo* cross-linking shows Alg44 interacts with AlgX. Cells were grown overnight in planktonic mode and incubated with cross-linker DSP (Lane 2, 3, 4 AB) or just DMSO (Lane 1 AB). Envelope fractions were isolated, detergent solubilised and subjected to affinity chromatography as mentioned in Materials and Methods. Elution fractions were probed with anti-Alg44 (A) or anti-AlgX (B) antibodies. A protein with an apparent molecular of 90 kDa was found to bind both anti-Alg44 (A) or anti-AlgX (B) antibodies and appeared only when Alg44-His expressing cells were used and treated with DSP (Lane 3, AB). This ~90 kDa protein was not detected or in cells producing wild type Alg44 (without histidine tag) (Lane 4, A B) or when no cross-linker was used (Lane 1, AB) or in cells lacking both AlgX and Alg44 and expressing only Alg44-His (Lane 2, AB).

Discussion

Here the role of individual subunits of the alginate polymerisation/secretion machinery in assembly and stability of the proposed multiprotein complex was investigated. One of the components, AlgK, is a periplasmic lipoprotein anchored to the inner leaflet of the outer membrane by its lipid moiety (86). This protein has multiple copies of the tetratricopeptide-repeat motif commonly found in proteins involved in the assembly of multiprotein complexes (86). To elucidate the role of AlgK in alginate biosynthesis an isogenic *algK* deletion mutant in the constitutive alginate overproducer *P. aeruginosa* PDO300 was generated. This mutant PDO300Δ*algK* failed to produce high molecular weight alginate (Fig. 1B); a phenotype consistent with a previous study using the *algK* mutant, FRD1100, of strain FRD1 (81). Interestingly, PDO300Δ*algK* produced almost no free uronic acid oligomers while FRD1100 produced significantly more (Fig. 2) (81). The lack of free uronic acids which was thought to be the product of alginate degradation in the periplasm by alginate lyase, indicated that in the absence of AlgK the alginate polymerisation might not occur. This would suggests that AlgK may play a role in the polymerisation of alginate or more likely play a role in the stability of the polymerases Alg8 and/or Alg44 in the inner membrane. This difference between our observations and those in FRD110 could be due to a difference in parent strain and method of mutant generation. In FRD1100 the *algK* ORF was replaced with a gentamycin resistance cassette containing a promoter which would elevate the transcription of downstream genes such as *algA* which is involved in alginate precursor (uronic acids) synthesis. In contrast, PDO300Δ*algK* has no extra promoter or marker present. The yield of free uronic acids produced by PDO300Δ*algK* was comparable to that of PDO300Δ*algX,* and PDO300Δ*alg44* suggesting that AlgK (and AlgX) have roles in alginate polymerisation, possibly through stabilising Alg8 and Alg44, the polymerase and co-polymerase of alginate synthesis, respectively (Fig. 2). This was supported by our mutual stability assays of the various mutants (PDO300Δ*algK*, PDO300Δ*alg44*, and PDO300Δ*algX*) which demonstrated that AlgK, Alg44 and AlgX were interdependent for stability, suggesting that these proteins might interact (Fig. 3A).

We have previously proposed a model of the assembly of alginate biosynthesis and secretion complex (Fig. 6 modified from chapter IV). In the current study mutual stability experiments were performed to identify potential protein-protein interactions

between subunits of the alginate biosynthesis machinery. The principle of mutual stability is that the deletion of a subunit would destabilise its interacting partner. To provide further experimental support beyond mutual stability analysis, pull-down and co-IP assays were used to demonstrate the interactions. The results of mutual stability analysis using mutant PDO300Δ*algE* (Fig. 3A lane 7) and that AlgK was required for the proper localisation of AlgE to the outer membrane (86) provided a strong basis to further explore the proposed AlgK-AlgE interaction. A co-IP assay was employed, under native conditions, using FLAG-tagged AlgE which provided strong evidence for an interaction between AlgK and AlgE (Fig. 4A). Similar to this, functional homologues of AlgE and AlgK, HmsH and HmsF respectively, of poly-*β*-1,6-N-acetyl-D-glucosamine (PGA) secretion system of *Yersinia pestis*, were found to interact (2). AlgK appears to be a weakly produced protein, as suggested by our inability to detect AlgK in cells grown in planktonic growth mode (Fig. 3A lane 12). Hence, to increase the likelihood of identifying potential interaction partners (AlgX, Alg44 or AlgE) of AlgK, pull-down assays from cells grown on solid media were performed; this biofilm mode of growth is characterized by increased alginate production and possibly the number of alginate biosynthesis complexes. Pull-down experiments using AlgK-His demonstrated that AlgK interacts with AlgX; a finding consistent with previous work (71) (Fig. 4B).

Mutual stability analyses using Δ*alg44* and Δ*algX* suggested an interaction between these components (Fig. 3 lane 5, 6, 9 & 10). Furthermore, the periplasmic C-terminal domain of Alg44 is similar to membrane fusion proteins of multi-drug efflux pumps and is thought to interact with the periplasmic and/or outer membrane components of the alginate biosynthesis machinery (108, 120, 138). To investigate this possibility, pull-down assay using Alg44-His were performed. Under native conditions, in the absence of crosslinker DSG no interaction partner was found suggesting that interactions of Alg44 are weak and/or transient or dependent on an intact membrane. However, in the presence of DSG a protein with an apparent molecular weight of ~90 kDa corresponding to the combined molecular weights of AlgX (49.9 kDa) and Alg44 (41.2 kDa) was detected using anti-Alg44 or anti-AlgX antibodies (Fig. 5A, 5B). This data suggested an interaction between Alg44 and AlgX. The interaction between Alg44-AlgX-AlgK explains why reduced amounts of free uronic acids were detected for the *algK* and *algX* mutant, respectively, because in both mutant the co-polymerase Alg44

was destabilised, which in turn impaired alginate polymerisation (Fig. 3A lane 9 & 11). Hence, no alginate was synthesized in the periplasm and no degradation by the alginate lyase could lead to free uronic acid formation.

Protein-protein interaction was proposed to occur between the putative polymerase (Wzy) and co-polymerase (Wzc) of the *E. coli* type I capsular polysaccharide biosynthesis system; however, experimental evidence for this interaction is still lacking (183). Wzy and Wzc can be considered analogues of Alg8 and Alg44 of the alginate biosynthesis machinery. Interestingly Alg44 and its interacting partners (AlgX-AlgK) were completely destabilised in PDO300Δ*alg8* (Fig. 3A lane 3). Alg44 is a putative co-polymerase and deletion of *alg44*, its cytoplasmic c-di-GMP binding PilZ domain or its periplasmic MFP domain resulted in loss of alginate production (108, 138). This provided evidence that Alg44 has an additional regulatory role in alginate production. The binding of c-di-GMP may induce conformational changes in Alg44 which may be required to activate Alg8. These findings and mutual stability analysis of the *alg8* mutant suggested that Alg44 and Alg8 may interact. In addition, *E. coli* BcsA protein, involved in cellulose polymerisation contains both a glycosyltransferase domain and a PilZ domain which further suggest that interaction between Alg8 and Alg44 might be required for polymerisation to occur (145, 185). However, future research using Alg8 pull-down assays will be required to support this hypothesis.

MucD is a negative regulator of alginate biosynthesis which has been shown previously to interact with AlgX which in turn interacts with AlgK. Deletion of *mucD* in PAO1 (PAO1Δ*mucD*) leads to increased transcription of *algD* operon and overproduction of alginate (14, 191). Based on this and our results it is difficult to conclude what structural role MucD might have in alginate production.

Previous studies have suggested that *algE* may have there own internal promoters (other than the promoter upstream of *algD*) (26). The presence of a faint band for AlgE and a strong band of AlgG in PAO1 (Fig. S1, 3A, lane 2) is in support of an independent expression of the two genes. Indeed, a recent publication described the existence of an internal promoters for *algI* and *algG,* suggesting that an independent regulation of *algG* expression controlled the degree of alginate epimerisation and hence its material properties (122). Although strain PAO1 is non-mucoid, alginate has been demonstrated to be produced by this strain, at low levels, through the use of highly sensitive

immunofluorescence microscopy (18). The elevated AlgG presence in PAO1 may result in the production of alginates containing a higher proportion of guluronic acid residues. Alternatively, this suggested that AlgG has other functions apart from its function in alginate production.

OM protein, OmpX, of *Y. pestis* has been described to play a role in adhesion (91). The role of AlgE in attachment to polystyrene microtitre plates was evaluated by using PAO1Δ*algE* in comparison to wild type PAO1. However, results suggested that AlgE does not play a role in attachment (data not shown).

In the present study, pull-down experiments, co-IP and mutual stability analysis provided evidence for the existence of a multiprotein complex constituting the alginate polymerisation and secretion machinery as well as resolved protein-protein interactions between individual subunit of this complex (Table 1). It was shown that OM protein AlgE interacts with periplasmic AlgK. Further AlgK was shown to interact with periplasmic AlgX which in turn interacts with inner membrane protein Alg44. These results support the existence of transmembrane multiprotein complex facilitating alginate polymerisation and secretion. Although it has been proposed that AlgI, AlgJ and AlgF form a complex but apparently they are not involved in alginate polymerisation or secretion (51). Based on the results obtained by mutual stability and protein-protein interaction assays we have proposed a model for the assembly of alginate biosynthesis machinery (Fig. 6).

Table 1. Proposed interactions between various proteins of alginate biosynthesis and secretion complex.

Protein	Proposed Interactions
Alg8	Alg44[a], AlgG[a]
Alg44	Alg8[a], AlgX[a,b]
AlgE	AlgK[a,b]
AlgX	Alg44[a], AlgK[a,c]
AlgK	AlgE[a], AlgX[a,b]
AlgG	Alg8[a]

[a] – Mutual stability data

[b] – Pull-down data or Co-immunoprecipitation

[c] – Past experimental data

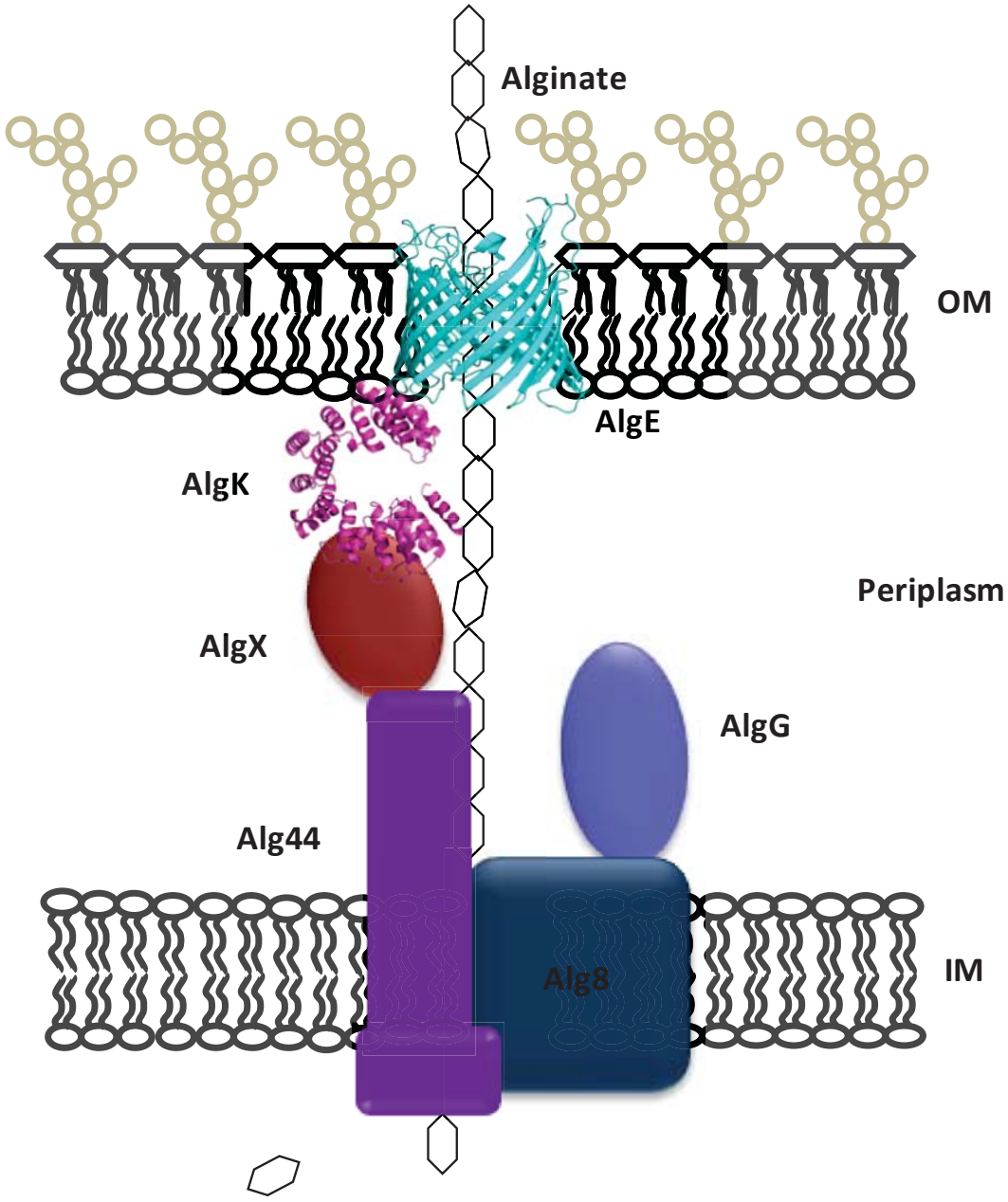


Fig. 6: Proposed model of the alginate polymerisation/secretion multiprotein complex. Based on results from mutual stability and pull-down assays, a model of the assembly for alginate polymerisation and secretion machinery was proposed. This model suggest that OM located AlgE interacts with periplasmic AlgK which in turns interacts with another periplasmic protein AlgX. Further AlgX interact with inner membrane Alg44 which may interact with Alg8 which in turn may interact with AlgG.

Acknowledgments

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Supplementry Material

Table S1. Bacterial strains and plasmids used in the study.

Strains or plasmids	Characteristics	source
Strains		
P. aeruginosa PAO1	Prototrophic wild type strain; Alg^-	(77)
P. aeruginosa PDO300	*mucA22* isogenic mutant derived from PAO1	(77)
PDO300Δ*algK*	Isogenic *algE* deletion mutant derived from PDO300	This study
PDO300Δ*alg8*	Isogenic *algE* deletion mutant derived from PDO300	(140)
PDO300Δ*alg44*	Isogenic *algE* deletion mutant derived from PDO300	(138)
PDO300Δ*algX*	Isogenic *algE* deletion mutant derived from PDO300	(61)
PDO300Δ*algE*	Isogenic *algE* deletion mutant derived from PDO300	(69)
PAO1Δ*mucD*	Isogenic *algE* deletion mutant derived from PDO300	(71)
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	(163)
Plasmids		
pEX100T	Ap^r Cb^r, gene replacement vector containing *sacB* gene for counterselection	
pEX100T:Δ*algE*ΩGm	Ap^r Cb^r Gm^r; vector pEX100T with SmaI-inserted *algE* deletion construct	(69)

pEX100T:Δ*algK*ΩGm	Ap^r Cb^r Gm^r; vector pEX100T with *EcoRV*-inserted *algK* deletion construct	This study
pEX100T:Δ*algX*ΩGm	Ap^r Cb^r Gm^r; vector pEX100T with *EcoRV*-inserted *algX* deletion construct	(61)
pPS865	Ap^r Gm^r; source of 1,100-bp *BamHI* fragment comprising *aacC1* gene flanked by Flp recombinase target site signal sequences	(74)
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(74)
pGEM®-T Easy	Ap^r, P_{lac}	Invitrogen
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	
pBBR1MCS-5: *alg44*	*HindIII-BamHI* fragment comprising *alg44* inserted	(138)
pBBR1MCS-5: *algE*	*HindIII-BamHI* fragment comprising *algE* inserted	(69)
pBBR1MCS-5: *alg44algX*	*HindIII* and *ClaI* fragment comprising *alg44* was ligated into pBBR1MCS-5:*algX* hydrolysed with same enzymes	
pBBR1MCS-5: *algK*	*HindIII-BamHI* fragment comprising *algK* inserted	This study
pBBRMCS-5: *algE*L6FLAG	*algE* fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 513^{th} bp of the ORF	(69)
pGEM-TEasy: P*algD*	-879 bp region, flanked by *PstI* and *BamHI*, relative to the *algD* open reading from was amplified and ligated	This study
pGEM-TEasy: P*algU*	-901 bp region, flanked by *XbaI* and	This study

	HindIII, relative to *algU* open reading frame was amplified and ligated	
mini-CTX-*lacZ*	Integration proficient vector	(75)
miniCTX:P*algK*	*algK* frgment with *algD* promoter cloned into chromosomal integration vector	This study
miniCTX:P*alg8*	*alg8* frgment with *algD* promoter cloned into chromosomal integration vector	This study
miniCTX:P*alg44*	*alg44* frgment with *algD* promoter cloned into chromosomal integration vector	This study
miniCTX:P*algX*	*algX* frgment with *algD* promoter cloned into chromosomal integration vector	This study
miniCTX:P*algE*	*algE* frgment with *algD* promoter cloned into chromosomal integration vector	This study
miniCTX:P*mucD*	*mucD* frgment with *algU* promoter cloned into chromosomal integration vector	This study

Table S2. Oligonucleotides used in this study.

Primers	**Sequence**
algKNF(EcoRV)	GATATCATGAAGATGCCCATCCTCCCTCC
algKNR(BamHI)	GGATCCCGATTTCCGGCCAGGACTGC
algKCF(BamHI)	GGATCCTACTACCTGGGGCAGATCTA
algKCR(EcoRV)	GATATCCTCATAGGCTTTCTGGCTCTTC
algK(upXout)	ACCCTGCTGAACAAGGCCGTGAC
algK(downXout)	GCGGGTTGACGGAACGGGAGCTG
algKN(HiSDNd)	ACCAAAGCTTAGGAGAGAAAGCATATGAAGAT
	GCCCATCCTCCCTCCCCTGC
algKC(BamHI)	GACGGATCCTCATAGGCTTTCTGGCTCTTCTTCG
algKC(6xhBamHI	GTCTGGGATCCGACTTAATGATGGTGATGGTGG
	TGTAGGCTTTCTGGCTCTTCTTC
	GTTGATCGGCGAG
Pser Up	CGAGTGGTTTAAGGCAACGGTCTTGA
Pser Down	AGTTCGGCCTGGTGGAACAACTCG
palgUXbaIR	TCTAGAGAAAGCTCCTCTTCGAACCTGGAGG
palgUHindIIIF	AAGCTTCGACCGCCACCCGCTGCGAC

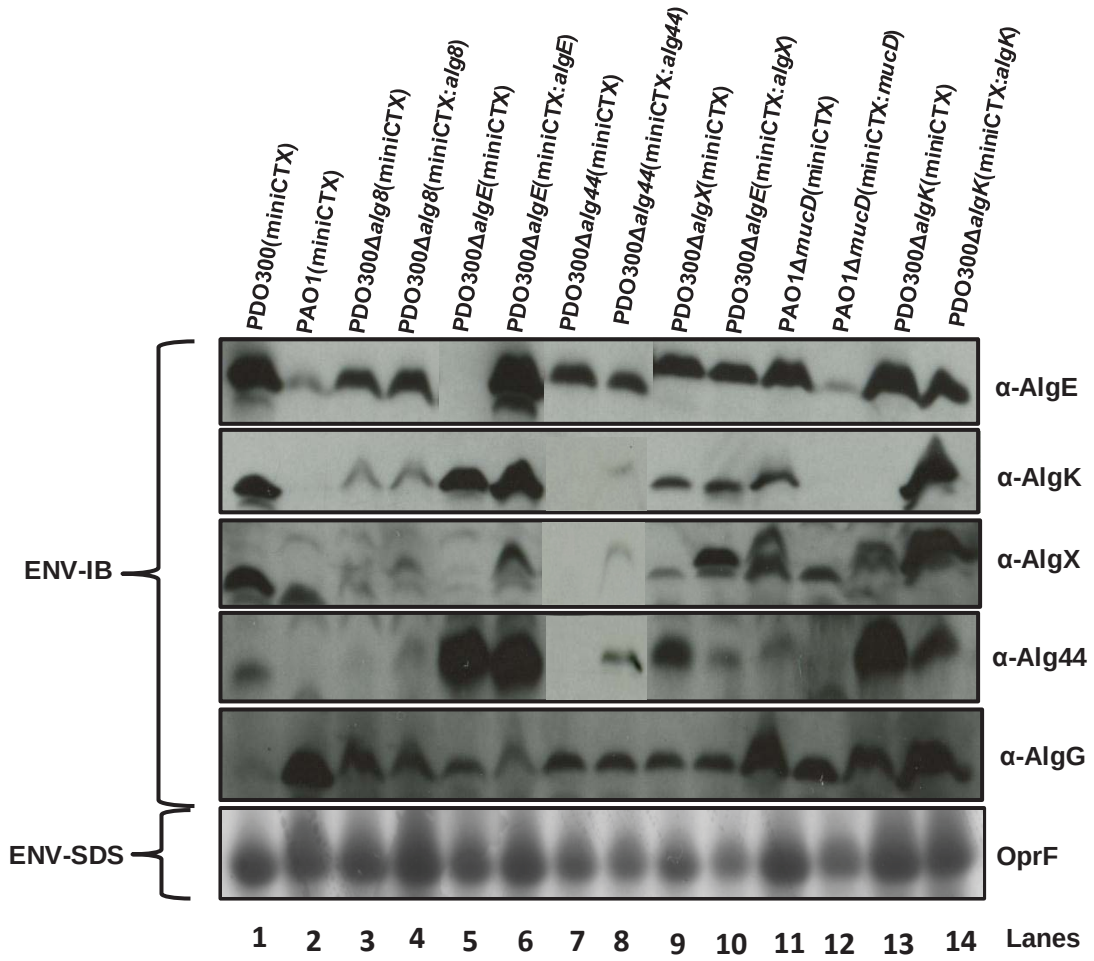

Fig. S1: Mutual stability analysis. Envelope fractions from PDO300, PAO1, knock-out mutants of *alg8*, *alg44*, *algE*, *algX*, *algK* and *mucD* and their *in cis* complemented strains, were isolated after 48 h growth on solid media and subjected to immunoblot analysis. The envelope fractions were probed for the presence or absence of various components of alginate polymerisation and secretion complex using antibodies as indicated (Lane 1-14). ENV: Envelop fraction, SDS-ENV: SDS-PAGE of envelope fraction. Only relevant parts of the blots were shown. Constitutively produced OM protein OprF was used as loading control.

Chapter VI

Final discussion and future directions

Final discussion

Chloroplast, mitochondria and gram-negative bacteria are surrounded by a remarkable structure referred to as outer membrane (OM). The OM of gram-negative bacteria is asymmetrical in that the inner leaflet of the membrane is composed of phospholipid whereas the outer leaflet is made up of lipopolysaccharides. The primary function of the OM is to serve as a permeability barrier and shield the cell from potentially cytotoxic agents. Although small molecular weight hydrophobic molecules can diffuse through the membrane, uptake of nutrients and secretion of important molecules is mediated by the proteins located in the OM (182).

Although the proteins located in the OM perform a variety of function, the pore-forming proteins allow the efficient transfer of important molecules across the membrane. The porin proteins located in the OM can be either general diffusion porins or substrate specific porins. The general diffusion porins allow the passage of small water soluble substances below 700 Daltons (141). Substrate specific porins, however, allow passage of substrates which are selected by the size of the pore and the properties of the amino acids lining the inside of the pore. Most of the pore-forming proteins found in the OM of *P. aeruginosa* are substrate specific. However, uptake of small water soluble molecules is mediated by members of the substrate specific Outer membrane carboxylic acid channel (Occ) family, formerly called the OprD family (41).

AlgE is a 54 kDa OM protein that was first detected in an alginate producing strain of *P. aeruginosa* whereas it was absent in the non-mucoid strains (58). It was subsequently shown that AlgE forms an anionic selective pore, suggesting its involvement in secretion of alginate which is a negatively charged polymer (131). Studies have shown that antibodies against AlgE were detected in CF patients making AlgE a potential target for drug design or vaccine development (133). The aim of the present study was to establish the role of AlgE in alginate biosynthesis and secretion. To achieve this, an isogenic knock-out mutant of *algE* was created in the alginate producing strain PDO300 of *P. aeruginosa*. Deletion of *algE* resulted in a loss of alginate production which indicated its involvement in alginate biosynthesis. To rule out the possibility that the deletion of *algE* had a polar effect on downstream genes the open reading frame (ORF) of *algE* was cloned onto the plasmid (pBBR1MCS-5:*algE*) which was subsequently introduced into PDO300Δ*algE*. The resulting strain PDO300Δ*algE*(pBBR1MCS-5:*algE*) produced alginate which further confirmed that lack of alginate production was

caused by the absence of AlgE. Previous studies have shown that deletion mutants of the periplasmic proteins AlgG, AlgX and AlgK resulted in the loss of alginate production but free uronic acids, which are the degradation products of alginate, were detected in the culture supernatant (80, 81, 143). It has been hypothesized that free uronic acids are produced when the integrity of periplasmic scaffold is compromised and alginate becomes exposed to the alginate degrading enzyme, alginate lyase, AlgL (82). Free uronic acid analysis of the *algE* mutant suggested that alginate was still being polymerised but was unable to be secreted and therefore it was degraded. These results indicate that AlgE does not have a specific role in alginate polymerisation but is involved in secretion (Chapter III).

Bioinformatics analysis suggested that AlgE forms a β-barrel structure with nine extracellular loops, eighteen transmembrane regions and eight periplasmic turns. To confirm the topology of AlgE, suggested by bioinformatics analysis, FLAG epitope (DYKDDDDK) insertion mutagenesis was performed. The rationale of this approach is that any insertion in the extracellular loops or periplasmic turns will be permissible whilst insertion into the β-strands, which could perturb their amphipathic nature, will not be permissible. This approach has been employed successfully to investigate the topology of OM proteins, for example, LamB of *E. coli* and OprH of *P. aeruginosa* (116, 134). Through this approach it was confirmed that AlgE has nine extracellular loops, and that extracellular loop 7 plays an important role in folding and/or the stability of the protein (69) (Chapter III). This was subsequently confirmed by the crystal structure of AlgE which shows that loop 7 is bent inside the barrel lumen and plays an important role in the stability and function of AlgE (184). Except for transmembrane regions 6 and 10 (β-strand 6 and β-strand 10), insertion of FLAG epitope into the transmembrane regions were not tolerated. However, insertions into β-strand 6 and 10 were not tolerated completely as M6F (insertion into β-strand 6) was not active and M10F (insertion into β-strand 10) was only active on solid media suggesting that insertion into these sites are only semi-permissible. Semi-permissibility of transmembrane regions has been reported for LamB where some of the insertions made in the transmembrane regions close to the periplasm turns or extracellular loops were tolerated to some extent (116). The detection of the M10F variant in OM isolated from cells grown on solid media suggests that the mode of growth can influence the activity of the BAM complex which is involved in the assembly and insertion of OM proteins. It

is worth noting that alginate production is induced upon growth on solid media (73) which may influence the assembly and insertion of AlgE variant, M10F, which is be required for the secretion of alginate (Chapter IV).

Insertion of FLAG epitopes into the periplasmic turns, T2F, T4F, T5F, T6F, T8F-1, T8F-2 and T8F-3 were tolerated and all the variants were detected in the OM and restored alginate production. Insertion into the periplasmic turns led to varying levels of alginate production. This suggested that periplasmic turns have a role either in the stability of AlgE through interaction with other components, or in the recognition and efficient secretion of alginate. This was further supported by the production of 100% free uronic acids by the variants with insertions in periplasmic turns T2, T4, T5 and T8-1. Deletion of *algE* affected the stability of AlgK and AlgX which were not restored when *algE* was expressed *in trans* (on plasmid) possibly because of disturbances in the stoichiometry of these proteins. To address this issue, *algE* and its variants with insertions into the periplasmic turns T2, T4, T5 and T6 were integrated into the PDO300Δ*algE* backbone. This ensured that there was only a single copy of the *algE* gene per genome under the control of its native *algD* promoter. Expression of *algE in cis* and its variants T2F, T5F, T6F restored the presence of AlgK and AlgX in the envelope which suggested that AlgE interacts with AlgK or AlgX. Interestingly, although the T4F variant of AlgE was detected in the OM it cannot restore AlgK or AlgX which indicates that this turn plays an important role in mediating interaction of AlgE with AlgK and/or AlgX. Deletion of *algE* also resulted in a reduction in the amount of Alg44 in the inner membrane and expression of *algE in cis* restored the Alg44 copy number. These results provide the first direct evidence of existence of a trans-envelope multiprotein complex required for the biosynthesis of alginate (Chapter IV). A reduced amount of T2F was detected in the OM which suggested that periplasmic turn 2 plays a role in the stability of AlgE possibly through interaction with other protein.

The crystal structure of AlgE reveals that the conserved and positively charged residues lining the lumen may have a role in efficient secretion of alginate. The proposed conduit for alginate secretion is lined with the highly conserved charged residues K47, R74, R129, R152, D162, N164, R353, R362, R459 and D485 as well as the less well conserved residues E130, R154, E189, D193, H364, R365, E368, R461 and R481. Using site-directed mutagenesis, the charges of these residues were either reversed or

the residues were converted to alanine. Except for one residue, R129, substitution of all of the positively charged residues resulted in a decrease in the amount of alginate produced. To further investigate this we created double and triple mutants. Two triple mutants, K47E+R353A+R459E and R74E+R362A+R459E, which significantly decrease the positive electrostatic field inside the AlgE lumen, showed the greatest reduction in alginate production. Substitution of two negativly charged residues D162 and D485 with alanine resulted in increased alginate production. These results strongly suggest that a positive electrostatic field inside the AlgE lumen is required for efficient secretion of negatively charged alginate (Chapter IV). Considering that AlgE is an export protein, not an enzymatic protein, any expectation of complete loss of functionality with site-specific mutagenesis without disrupting its fold would be unrealistic. Similarly for LamB in *E. coli*, the substrate specific maltoporin, the complete loss of functionality was achieved only by mutating all of the six residues involved in maltose uptake (36).

Mutual stability analysis using PAO*1mucD* and isogenic knock-out mutants of *alg8*, *alg44*, *algE*, *algX*, *algK* in PDO300 suggested that Alg8 interacts with the periplasmic epimerase, AlgG, and inner membrane "co-polymerase", Alg44, which in turn interacts with periplasmic AlgX and/or AlgK. In a pull-down assay, AlgX co-purified with Alg44-His which suggests an interaction between these components. Interaction between AlgX and AlgK has been reported previously and was further confirmed by mutual stability analysis and AlgK-His pull down. Previous reports and mutual stability analysis suggested an interaction between AlgK and the OM localized AlgE (86, 184). To investigate further a co-immunoprecipitation assay using FLAG tagged AlgE was carried out which confirmed an *in vivo* association of AlgE and AlgK. Based on mutual stability and pull-down assays interactions between AlgG-Alg8-Alg44-AlgX-AlgK-AlgE were proposed (Chapter V).

In PAO1 while no other protein was detected, the presence of AlgG and a faint band for AlgE suggested the existence of internal promoters for these proteins. This agrees with a previous study which suggested a weak internal promoter for *algE* gene (Chapter V) (26). It has been proposed that OM proteins OmpX and HmsH of *Yersinia pestis* play a role in cell adhesion and biofilm formation respectively (2, 91). However a solid surface attachment assay using an *algE* knock-out strain and its complemented strain did not show a significant difference in attachment. As our manuscript was under preparation a

paper was published which confirms the existence of an internal promoter for AlgG. However this study was conducted in an alginate producing strain and concluded that the internal promoter would help regulate the level of AlgG in the cell and therefore the degree of epimerisation of alginate and its material properties (122). The presence of AlgG in PAO1, which produce only a small amount of alginate as detected by immunoflourescence microscopy, might suggest that AlgG have other functions apart from acting as an epimerase in alginate biosynthesis. Future research will endeavour to assigns functionality to AlgG in PAO1.

Future directions

Outer membrane proteins like HmsH are not only required for the secretion of polysaccharide but they also play a role in the formation of biofilms. A recent study has also suggested that OM proteins play an important role in biofilm formation. AlgE has large extracellular loops, for example, loop 5 and 6 and their function is not clear. It would be interesting to investigate if extracellular loops play a role in adhesion to biotic surfaces. Flow chamber assays can be used to investigate if AlgE play an alginate independent role in biofilm formation.

Although we know many aspects of alginate regulation and polymerisation, the number and sub-cellular localization of alginate biosynthesis machinery remains elusive. Immunofluorescence microscopy using anti-AlgE antibodies can be used to investigate the surface localization of AlgE which would give an idea where in the cell the alginate biosynthesis complex is located. Furthermore immuno-gold labelling using primary antibodies against the proteins of the alginate biosynthesis complex would give a much more detailed picture of the localization and number of the complexes.

Mutual stability analysis suggests that AlgE may also interact with AlgX. However this interaction was not confirmed under the experimental conditions used. By varying buffer composition and applying different cross linkers may increase likelihood of detecting an AlgX-AlgE interaction. Interaction between Alg8 and AlgG has also been proposed which can be investigated using pull-down assays. Although in our study Alg8 was not found to be associated with Alg44, literature does supports the possibility of interaction between these components. Different experimental conditions and strategies should be employed to investigate this further.

The presence of internal promoters for *algE* and *algG* has been suggested. Identification of the transcription start site and regulation of these promoters under specific growth conditions would better elucidate the function of these proteins. High level expression of AlgG in PAO1 suggests that the alginate produced by PAO1 has different level of epimerisation which would affect its material properties. Alginates with different materials properties may also exhibit different resistance to antibiotics or human immune responses and architecture of biofilms.

The work proposed here will assist in developing and enhancing our knowledge and understanding the mechanism of alginate production which in turn will assist in combating what is the leading lethal pathogen in cystic fibrosis patients.

Somewhere, someting inceredible is waiting to be known. (Carl Sagan)

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