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Molecular Mechanism of Alginate Polymerisation and Modifications in
Pseudomonas aeruginosa

A thesis presented in partial fulfilment of the requirements for degree

of

Doctor of Philosophy

in

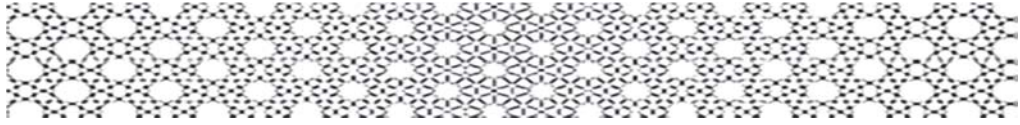
Microbiology

at Massey University

New Zealand

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2015



In Loving Memory of My Mom

Pari



*“Science is a way of thinking much more
than it is a body of knowledge”*

Carl Sagan



Abstract

Pseudomonas aeruginosa is an ubiquitous opportunistic human pathogen in immunocompromised patients. It is of particular relevance to cystic fibrosis (CF) patients where it frequently causes chronic bronchopulmonary infection and is the leading cause of morbidity and mortality. The decline in lung function is caused by the emergence of a mucoid variant showing excessive production of the exopolysaccharide, alginate. The alginate-containing biofilm matrix of this mucoid variant protects *P. aeruginosa* from the immune system and antibiotics.

Here the alginate biosynthesis/modification/secretion multiprotein complex was investigated with regard to protein-protein interactions constituting the proposed multiprotein complex and the molecular mechanisms underlying alginate polymerisation and modifications. This study sheds light on the structure and function of various alginates from a material property and biological function perspectives. The binary interactions of AlgK-AlgE, AlgX-Alg44, AlgK-Alg44 and Alg8-Alg44 were identified proposing a new model for this multiprotein complex organisation. Protein-protein interactions were found to be independent of c-di-GMP binding to PilZ_{Alg44} domain. C-di-GMP-mediated activation of alginate polymerisation was found to be different from activation mechanism proposed for cellulose synthesis. This study showed that alginate polymerisation and modifications were linked. It was shown that the molecular mass of alginate was reduced by epimerisation, while it was increased by acetylation. It was determined that previously characterized proteins AlgG (epimerase) and AlgX (acetyltransferase) have mutual auxiliary and enhancing roles. Biofilm architecture analysis showed that acetyl groups lowered viscoelasticity of alginates and promoted cell aggregation, while nonacetylated polymannuronate alginate promoted stigmergy. Experimental evidence was provided that Alg44 boosted acetylation while the periplasmic domain of this protein was critical for protein stability and regulation of alginate modifications. Full-length Alg44 was purified and it was found to be a dimer in solution. Overall, this study sheds new light on the arrangement of the proposed alginate biosynthesis/modification/secretion multiprotein complex. Furthermore, the activation mechanism and the interplay between polymerisation and modification of alginate were elucidated and new functions and interactive role of alginate-polymerising and-modifying subunits were further understood.

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 or submitted or drafted for publication in internationally peer-reviewed journals. Publications have not been arranged in chronological order.

Chapter II

Hay ID, Ur Rehman Z, Moradali MF, Wang Y, Rehm BHA (2013). Microbial Alginate Production, Modification and its Applications. *Microbial Biotechnology* **6**: 637–650.

Chapter III

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Chapter IV

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Chapter V

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Chapter VI

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Contributions Moradali MF made to publications are as follows

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

Chapter III: This review was drafted by I.D.H, W.Y, M.F.M and Z.U.R and finalised by B.H.A.R

Chapter IV: Chromosomal integration of all the genes was designed and made by Z.U.R. Generation of PDO300 Δ *algK* knock-out and its complementation was done by Y.W. AlgE co-immunoprecipitation was done by Z.U.R. AlgK pull-down was done by Y.W and PDO300 Δ *alg44* Δ *algX* mutant was created by I.D.H. This mutant was re-confirmed and transformed with pBBR1MCS-5:*alg44-6his* by M.F.M. *In vivo* chemical cross-linking and Alg44 pull-down assay was performed by M.F.M. Manuscript was drafted by Z.U.R, Y.W and M.F.M and I.D.H and finalised by B.H.A.R.

Chapter V: Generation of double-gene knockout mutants in *alg8/alg44*, *algG/algX* and *algG/alg44*, single-gene knockout mutant in *algG* and re-confirming single-gene knockout mutants in *alg8*, *alg44* and *algX*, construction of plasmids for complementing these mutants, chromosomal integration of the genes and site-specific mutations and deletions for producing Alg8, Alg44, AlgX and AlgG variants, *in vivo* detection of protein-protein interaction network, isolation of cytoplasmic membrane and general protein analysis and all other assessments were performed by M.F.M. ¹H-NMR analysis was done and interpreted by I. D and M.F.M. SEC-MALLS analysis was performed by I.S and M.F.M. Setting up and analysis of biofilms was performed by M.F.M and S.G. Manuscript was mainly drafted by M.F.M. and finalized by B.H.A.R.

Chapter VI: All of the experimental work was done by M.F.M. Manuscript was drafted by M.F.M and finalised by B.H.A.R.

DNA sequencing was provided by external services.

This is to certify that above mentioned work was conducted by M. Fata Moradali.

Signature Date

Prof. Bernd H.A. Rehm

Signature Date

M. Fata Moradali

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LIST OF ABBREVIATIONS

°C	Degree Celsius
Ap	Ampicillin
BSA	Bovine serum albumin
Cb	Carbenicillin
Δ	Delta (deleted)
DMSO	Dimethyl sulfoxide
D ₂ O	Deuterium oxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
EtOH	Ethanol
EDTA	Ethylenediaminetetraacetic acid
g	gravity/gram
Gm	gentamycin
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Daltons
λ	Lambda (wavelength or type of phage)
LB	Luria-Bertani (broth)
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PIA	Pseudomonas isolation agar
PPI	Protein-protein interaction
RNAase	Ribonuclease
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate gel electrophoresis
TBE	Tris-Borate-EDTA buffer v/vii
Tc	Tetracycline
TE	Tris-EDTA buffer
T _m	Primer melting temperature
Tris	Trishydroxymethylaminomethane
vol/vol	Volume per volume
wt/vol	Weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter I

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen which can become life-threatening in immunocompromized patients. It is the leading cause of morbidity and mortality in cystic fibrosis patients when it colonizes lung due to switching from non-mucoid phenotype to mucoid. Then, it forms structured biofilms which protect bacteria against immune system and antibiotic treatments. The biofilm of *P. aeruginosa* consist of bacterial cells embedded in a complex matrix predominantly composed of alginate (1, 2).

Alginates are anionic exopolysaccharides composed of variable proportions of 1,4-linked β -D-mannuronic acid (M) and its C5 epimer α -L-guluronic acid (G). Alginate has been identified to be necessary for maturation and development of *P. aeruginosa* biofilm structure and it is known as a virulent factor due to its ability to reduce uptake and diffusion of antibiotics, interfere with polymorphonuclear leukocyte (PMN)-mediated killing of mucoid strains (3, 4) and scavenging free radicals released by triggered mechanisms in the immune system, leading bacterial protection from innate immune responses (5).

Alginate is also produced by algae and by the bacterial species belonging to the *Azotobacter* genus. Mucoid strains of the soil bacterium *A. vinelandii* produce alginate which is essential for encystment process in the soil. The main difference between alginates from *P. aeruginosa* and *A. vinelandii* is that the latter produces alginates with consecutive G residues (G-blocks) (6).

Alginates exhibit unique gel-forming properties suitable for numerous medical and industrial applications (7, 8). The alginate structure strongly impacts its material properties. The alginate derived from *P. aeruginosa* is naturally acetylated and lacks GG-blocks, while algal alginates lack acetyl groups, but have GG-blocks. These differences bring different physiochemical properties to the respective alginates (7). Hence, development of bioengineering approaches to control the alginate structure will enable production of alginates with new material properties towards novel applications. For many years *P. aeruginosa* has been the model organism to study various aspects of alginate biosynthesis such as polymerisation, epimerisation, acetylation, secretion and regulation. The genetics and biochemical pathways underlying alginate production will be reviewed in more detail in the next chapter. Briefly, thirteen proteins are involved in the biosynthesis of alginate and except for AlgC, their encoding genes are clustered in the alginate biosynthesis operon (*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, *algA*) (9, 10). Except for soluble cytoplasmic proteins AlgA, AlgC and

AlgD which are responsible for providing the activated nucleotide sugar precursor, GDP-mannuronic acid, proteins encoded by the operon are proposed to constitute an envelope-spanning multiprotein complex. Two cytoplasmic membrane-anchored proteins, the glycosyltransferase, Alg8, and the proposed co-polymerase, Alg44, are necessary for alginate polymerisation (11-13). The cytoplasmic PilZ domain of Alg44 binds c-di-GMP which is a key regulator of bacterial physiology, also regulating alginate polymerisation through an unknown mechanism (14). The MucR sensor protein, a diguanylate cyclase (DGC)/phosphodiesterase (PDE) embedded in the cytoplasmic membrane was proposed to provide c-di-GMP pool for Alg44 (15).

Translocation and guiding of nascent alginate across the periplasm for secretion via the outer membrane protein AlgE is mediated by a proposed periplasmic scaffold which mainly consists of multifunctional proteins, including AlgG, AlgX and AlgK (16-21). This event is coupled with modification processes, including O-acetylation and epimerisation. O-acetylation is independently catalyzed by AlgJ and AlgX (22), while the acetyl group donor is provided by AlgI and AlgF (23, 24). The AlgG epimerase converts M residues to G residues in the nascent alginate chain. It was also suggested that if alginate is misguided into the periplasm, it is degraded by the periplasmic AlgL lyase (25). There are several levels of regulation mechanisms for alginate biosynthesis under the physiological conditions which will be covered in detail in chapter 3.

Many proteins of proposed multiprotein complex including AlgX, AlgG, AlgJ, AlgK, AlgE and AlgL have been crystallized and their structure-function relationship have been characterised. AlgG was identified as a processive enzyme with a long right-handed parallel β -helix with an elaborate lid structure. The mechanism of the epimerase reaction (i.e. conversion of M to G residue) by AlgG was proposed to be based on the β -elimination reaction of polysaccharide lyases (26). AlgX was revealed to be a two-domain protein. The N-terminal domain of AlgX has similar structure to AlgJ, both with structural homology to members of the SGNH hydrolase superfamily, while the C-terminal domain of AlgX has a carbohydrate-binding module that binds to alginate chain. These two O-acetyltransferases play independent roles in O-acetylation of alginate (22, 24). Also, the structural basis for alginate secretion was understood on the basis of AlgE and AlgK structure-function characterisation. AlgE and AlgK were suggested to form a dynamic complex for facilitating alginate secretion through the open pore of AlgE localized in outer membrane (19, 27, 28).

Thesis Aims:

The overall aims of this thesis are:

- To provide further insights into protein-protein interactions involved in constituting alginate polymerisation/modification/secretion machinery complex.
- To investigate alginate polymerisation mechanisms and involved protein-protein interactions.
- To investigate further functional and structural role of Alg44 protein and associated PilZ domain upon binding to second messenger c-di-GMP.
- To unravel molecular mechanism of alginate polymerisation mediated by second messenger c-di-GMP.
- To unravel functional relationships of alginate polymerisation and modifications including acetylation and epimerisation.

Thesis Findings:

Chapter II of this thesis contains a literature review on bacterial alginates, the molecular mechanisms of biosynthesis, current and potential applications of alginates, including bacterially produced or modified alginates for high value applications where defined material properties are required. This chapter was published in the international peer reviewed journal *Microbial Biotechnology*.

Chapter III of this thesis contains a review of the most recent findings about genetics and regulation of bacterial alginate production. It is composed of a mini review which was published in the international peer reviewed journal *Environmental Microbiology*.

Chapter IV of this thesis provides new insight into assembly of the alginate biosynthesis machinery in *Pseudomonas aeruginosa*. Overall experimental evidence was provided for the existence of a multiprotein complex required for alginate polymerisation and secretion. This chapter was published in the international peer reviewed journal *Applied and Environmental Microbiology*.

Chapter V of this thesis provides further insights into assembly of the alginate biosynthesis machinery in *Pseudomonas aeruginosa* and presents the first findings of

molecular mechanism of alginate polymerisation upon binding to c-di-GMP. Also, the functional and structural relationships of Alg8 (polymerase), Alg44 (co-polymerase), AlgG (epimerase) and AlgX (acetyltransferase) and the correlation of alginate polymerisation and modifications were unravelled. It also demonstrates the production of various alginates with respect to composition and physiochemical properties, followed by the analysis of their impact on biofilm architecture. This chapter was published in the international peer reviewed journal mBio.

Chapter VI of this thesis presents new insights into the functional and structural role of periplasmic domain of Alg44 and homologous and heterologous production of Alg44 dimer. This chapter is drafted for publishing.

Chapter II

Microbial Alginate Production, Modification and its Applications

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Abstract

Alginate is an important polysaccharide used widely in the food, textile, printing and pharmaceutical industries for its viscosifying and gelling properties. All commercially produced alginates are isolated from farmed brown seaweeds. These algal alginates suffer from heterogeneity in composition and material properties. Here, we discuss alginates produced by bacteria; the molecular mechanisms involved in their biosynthesis; and the potential to utilize these bacterially produced or modified alginates for high value applications where defined material properties are required.

Introduction

Alginates are polysaccharides composed of variable ratios of β -D-mannuronate (M) and its C-5 epimer α -L-guluronate (G) linked by 1–4 glycosidic bonds (Fig. 1). Alginates were first isolated from brown seaweeds in the 1880s, and its commercial production begun in the early 20th century. Alginate can be produced by various genera of brown seaweed and two genera of bacteria, *Pseudomonas* and *Azotobacter*. The ratio of M and G residues and thus the material properties vary depending on the source of the alginate. Its unique physical properties enable it to be used as a stabilizer, viscosifier and gelling agent in the food, beverage, paper, printing and pharmaceutical industries. Industrial production of alginate is estimated to be at least 30,000 metric tons annually with all of that coming from farmed brown seaweed, primarily from the genera *Laminaria* and *Macrocystis*. In these seaweeds, alginate plays a structural role and constitutes up to 40% of the dry matter of the plant; it is thought to play a role analogous to cellulose in terrestrial plants (29, 30). Recently, the desirable material properties as well as its apparent biocompatibility (31) has led to it being used increasingly in the medical, pharmaceutical and biotechnology industries for applications such as wound dressings (32); the encapsulation or controlled release of drugs, enzymes or cells; or as a matrices for tissue engineering (33, 34).

Microbial biosynthesis of alginate

Two genera of bacteria have been shown to secrete alginate, *Pseudomonas* and *Azotobacter*. Most of the research into the molecular mechanisms behind bacterial alginate biosynthesis has been conducted on the opportunistic human pathogen *Pseudomonas aeruginosa* or the soil dwelling *Azotobacter vinelandii*. Although these two genera utilize very similar molecular mechanisms to produce alginate, in nature, they secrete alginate for different purposes with different material properties: Some *P. aeruginosa* strains (known as mucoid strains) can secrete copious amounts of alginate to aid in the formation of thick highly structured biofilms (35, 36), whereas *Azotobacter* produces a stiffer alginate (with typically a higher concentrations of G residues) which remains closely associated with the cell and allows the formation of desiccation resistant cysts (37). The genes involved in alginate biosynthesis are virtually identical between *Pseudomonas* and *Azotobacter* though their regulation is slightly different. All but one of the core genes involved in alginate biosynthesis are contained within a single 12-gene operon initially described by Chitnis and Ohman (10): *algD*, *alg8*, *alg44*, *algK*,

algE (*algJ*), *algG*, *algX*, *algL*, *algI*, *algJ* (*algV*), *algF* and *algA* (*Pseudomonas* gene names are shown with the corresponding *Azotobacter* gene names in parentheses). The genes are under the tight control of a promoter upstream of *algD* (38, 39), although there is some evidence to suggest that alternative internal promoters exist within the operon (40, 41). The gene *algC* is not located within the operon and is also involved in rhamnolipid and lipopolysaccharide biosynthesis (42-44). In addition to these 13 core genes involved in alginate biosynthesis, many more have been identified and are summarized in Table 1. The steps of alginate biosynthesis can be loosely divided into four steps: precursor synthesis, polymerisation, periplasmic modification/transit and export.

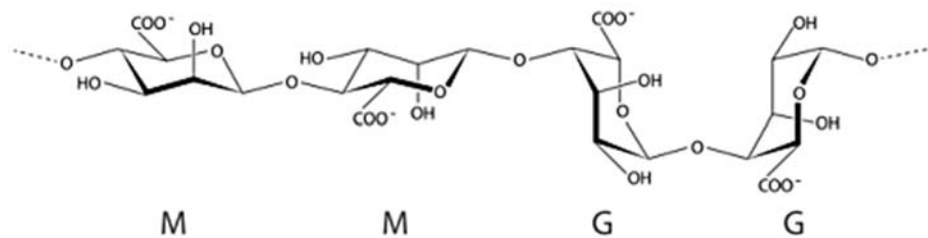


Fig. 1. Chemical structure of alginate.

M – mannuronate residues, G – guluronate residues.

Table 1. Proteins involved in alginate biosynthesis.

Protein	Description	Subcellular location	Reference
Core biosynthesis			
AlgA	Precursor synthesis. Phosphomannose isomerase/GDP-mannose pyrophosphorylase.	Cytosol	(Shinabarger <i>et al.</i> , 1991b)
AlgC	Precursor synthesis. Phosphomannomutase. PDB: 1P5G	Cytosol	(Ye <i>et al.</i> , 1994)
AlgD	Precursor synthesis. GDP-mannose dehydrogenase. PDB: 1 MV8	Cytosol	(Tatnell <i>et al.</i> , 1994)
Alg8	Polymerization. Proposed glycosyltransferase/polymerase.	IM	(Remminghorst <i>et al.</i> , 2009)
Alg44	Polymerization and post transcriptional regulation, c-di-GMP binding and response.	IM	(Remminghorst and Rehm, 2006a)
AlgK	Export/structural role. Lipoprotein, Stabilizes AlgE in OM. PDB: 3EB4	Associated with periplasmic side of OM	(Kaeski <i>et al.</i> , 2010)
AlgE	Export. OM porin. Named AlgJ in <i>Azotobacter</i> . PDB: 3RBH	OM	(Whitney <i>et al.</i> , 2011)
Modification			
AlgG	M-G epimerization. Mannuronan C-5-epimerase	Periplasm	(Franklin <i>et al.</i> , 1994)
AlgL	Alginate lyase. Control MW, clear alginate from the periplasm.	Periplasm	(Jain and Ohman, 2005)
AlgI	O-Acetylation	IM	(Franklin and Ohman, 2002)
AlgJ	O-Acetylation. Named AlgV in <i>Azotobacter</i>	IM	(Franklin and Ohman, 2002)
AlgF	O-Acetylation	Periplasm	(Franklin and Ohman, 2002)
AlgX	O-Acetylation. Structural role. Sequesters MucD. Structural role. PDB: 4KNC	Periplasm	(Gutsche <i>et al.</i> , 2006; Riley <i>et al.</i> , 2013)
AlgE1-E7	<i>Azotobacter</i> extracellular Mannuronan C-5-epimerases. PDB: 2PYG	Extracellular	(Ertresvåg <i>et al.</i> , 2009)
PA1167	Alginate lyase (polyguluronate lyase). PDB: 1VAV	Unknown	(Yamasaki <i>et al.</i> , 2004)
Regulation			
AlgU (AlgT, σ^{24})	Alternative σ factor homologous to <i>E. coli</i> σ^E global stress response factor. Positive regulator	Cytosol	(Xie <i>et al.</i> , 1996)
MucA	Anti σ factor. Negative regulator	IM	(Xie <i>et al.</i> , 1996)
MucB	Stabilizes MucA. Negative regulator	Periplasm	(Cozartlyan and Sauer, 2009)
MucC	Unclear regulatory role	Periplasm/IM	(Boucher <i>et al.</i> , 1997a)
MucD	Homologous to <i>E. coli</i> serine protease DegP. Negative regulator. Associated with Alginate complex. Negative regulator	Periplasm	(Wood and Ohman, 2006; Hay <i>et al.</i> , 2012)
AlgW	Homologous to <i>E. coli</i> serine protease DegS. Cleaves MucA. Positive regulator	IM	(Cozartlyan and Sauer, 2009)
MucP	Homologous to <i>E. coli</i> RseP protease. Positive regulator, cleaves MucA	IM	(Qiu <i>et al.</i> , 2007)
Pic	Protease. Positive regulator, cleaves MucA	Periplasm	(Wood <i>et al.</i> , 2006)
MucE	Positive regulator, activates AlgW	OM/Periplasm	(Qiu <i>et al.</i> , 2007)
ClpX/ClpP/ClpP2	Cytoplasmic proteases. Positive regulators, cleave MucA	Cytoplasm	(Qiu <i>et al.</i> , 2008)
AlgR	Two-component regulator (Cognate sensor is AlgZ/FimS). Positive regulator, binds to 3 regions in the <i>algD</i> promoter	Cytoplasm	(Ma <i>et al.</i> , 1998)
AlgB	NtrC-Family, two-component regulator (Cognate sensor is KlnB). Positive regulator, binds to one region in the <i>algD</i> promoter	Cytoplasm	(Ma <i>et al.</i> , 1998)
AmrZ	Arc-like DNA-binding protein. Positive regulator, binds to one region in the <i>algD</i> promoter (originally named AlgZ). PDB: 3OOQ	Cytoplasm	(Baynham and Wozniak, 1996; Baynham <i>et al.</i> , 2006)
AlgQ (AlgR2)	Positive regulator of nucleoside diphosphokinase, necessary for the formation of GDP-mannose	Cytoplasm	(Kim <i>et al.</i> , 1998)
AlgP (AlgR3)	Histone-like protein required for normal alginate expression, but does not appear to bind <i>algD</i> promoter	Cytoplasm	(Kato <i>et al.</i> , 1990)

Alginate precursor synthesis

The formation of the activated precursor guanosine diphosphate (GDP)-mannuronic acid is a well characterized process and is summarized in Fig. 2. It involves a series of cytosolic enzymatic steps feeding in to the membrane bound alginate polymerisation machinery. Synthesis starts with the entry of six carbon substrates into the Entner–Doudoroff pathway, resulting in pyruvate, which is channelled towards the tricarboxylic acid cycle. Subsequently, oxaloacetate is converted to fructose-6-phosphate via gluconeogenesis (45, 46). Three well-characterized enzymes, AlgA, AlgC and AlgD, catalyse the four subsequent biosynthesis steps to convert fructose-6-phosphate to GDP-mannuronic acid. First, the conversion of fructose-6-phosphate to mannose-6-phosphate is catalysed by the phosphomannose isomerase activity of the bifunctional protein AlgA (47). Then, AlgC (phosphomannomutase) converts mannose-6-phosphate to mannose-1-phosphate (48) followed by the conversion to GDP-mannose which is catalysed by the GDP-mannose pyrophosphorylase activity of AlgA via the hydrolysis of GTP (49). Interestingly, this AlgA catalysed step favours the reverse reaction, but the pull of the subsequent AlgD catalysed step shifts the reaction towards GDP-mannose production. The final step is catalysed by AlgD (GDP-mannose dehydrogenase) and is irreversible resulting in GDP-mannuronic acid, which is substrate for the alginate polymerisation machinery. The AlgD catalysed oxidation step is thought to be a key rate-limiting reaction in the alginate synthesis pathway (50-52).

Polymerisation

Polymerisation and translocation are relatively poorly understood processes in alginate biosynthesis. Disruption mutagenesis shows that at least two proteins are required for polymerisation: the inner membrane (IM) proteins Alg8 and Alg44 (53, 54). Disruption of the alginate biosynthesis genes generally either results in one of three phenotypes: no loss of alginate production (AlgI, AlgJ and AlgF) (23); loss of alginate production but release alginate fragments due to the activity of a periplasmic alginate lyase (AlgX, AlgG, AlgK and AlgE) (16, 55-57); or complete loss of alginate production and no alginate fragments, as is the case for Alg8 and Alg44 (53, 54, 58). Bioinformatic analysis suggests that Alg8 is the best candidate for a polymerase. It is predicted to be a glycosyltransferase (family-2 GT), which catalyses the transfer of a sugar molecule from an activated donor to an acceptor molecule (e.g. a growing carbohydrate chain). In accordance with functionally similar transmembrane glycosyltransferases such as

AcsAB (cellulose synthase) and Ch1 (chitin synthase), Alg8 showed a predicted structure with several transmembrane domains flanking a long cytoplasmic loop accommodating conserved motifs and catalytic residues. Site-directed mutagenesis of these predicted catalytic residues resulted in loss of alginate biosynthesis. Further experimental support for the direct involvement of Alg8 in alginate polymerisation is the observation that overexpression of Alg8 led to overproduction of alginate causing a supermucooid phenotype (36). This overproduction would seem to suggest that the polymerisation reaction catalysed by Alg8 is a bottleneck in the biosynthesis pathway. Interestingly, in-vitro polymerisation experiments showed that the entire cell envelope (IM and outer membrane plus associated proteins) was required for polymerisation, suggesting that Alg8 requires other proteins for function (54, 58, 59). The specific role Alg44 plays in polymerisation and the mechanisms involved remain unclear, but Alg44 is thought to play an indirect role. Similar to *alg8*, deletion of *alg44* gene resulted in no alginate polymerisation, while its overexpression led to overproduction of alginate. Alg44 is predicted as a multidomain protein which consists of a cytoplasmic PilZ domain, a transmembrane region and a periplasmic domain which shows homology to the membrane fusion protein MexA, a membrane-bridging protein involved in the multidrug efflux system of *P. aeruginosa* (53, 58). The periplasmic membrane fusion protein domain suggests that Alg44 may play a structural role in bridging the membrane bound polymerase to the periplasmic and outer membrane components facilitating the transit, modification and secretion of alginate. The cytosolic PilZ domain of Alg44 has been shown to bind the bacterial secondary messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) and is essential for alginate biosynthesis (60). Currently, it is unclear how the binding of c-di-GMP by Alg44 is conveyed to the polymerase (Alg8). Similar c-di-GMP-dependent carbohydrate polymerisation processes have been observed in various other systems, and these can give us clues as to how this mechanism function here (9, 61-63). In one recent study, it was shown that binding of c-di-GMP to the BcsA subunit of the cellulose synthase causes local conformational changes allowing UDP-glucose to access the catalytic site (64).

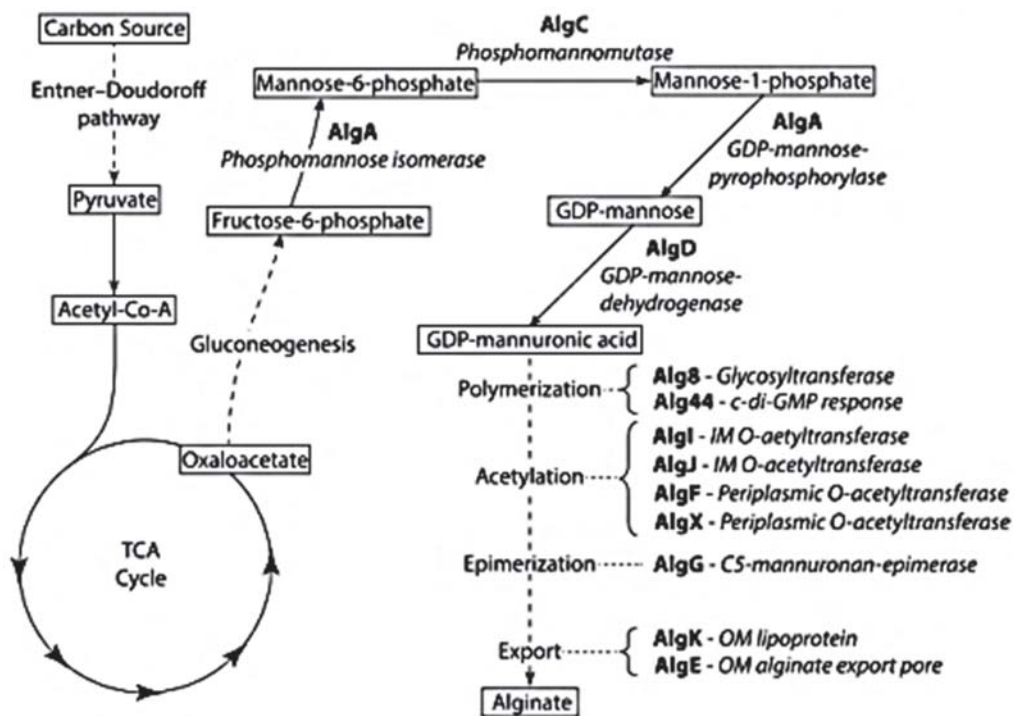


Fig. 2. Overview of bacterial alginate biosynthesis.

Periplasmic translocation and modification

After polymerisation, the nascent alginate chain (poly-M) is translocated across the periplasm by a putative multiprotein scaffold consisting of at least the periplasmic proteins AlgX, AlgG and AlgK (16, 55, 65). These proteins are thought to guide the alginate chain through the periplasm while protecting it from degradation by the periplasmic alginate lyase, AlgL. Intriguingly, it has been proposed that AlgL itself also contributes to the formation of a stable periplasmic scaffold (66). When components of the periplasmic scaffold are missing, alginate chain leaks into the periplasm where it is degraded by AlgL, releasing free uronic acid oligomers.

Initially, it seems somewhat counterintuitive to find an alginate degrading protein, AlgL, encoded within the alginate biosynthesis operon. Disruption of the *algL* gene in mucoid strains or mutation of the catalytic residues has proved to be difficult, often resulting in non-viability, or loss of mucoidity because of secondary mutations turning off alginate production. This suggests that both AlgL and its lyase activity are required for viability in mucoid strains, presumably serving a maintenance role by degrading misguided alginate trapped in the periplasm. AlgL may also actively control the length

of the polymer as well as contribute to periplasmic translocation (18, 25, 66). Recently, the details of the reaction catalysed by AlgL from *P. aeruginosa* were characterized. AlgL specifically cleaves the alginate chain via beta elimination, producing mannuronic acids with unsaturated non-reducing ends; the initial steps of this reaction are similar to epimerisation. It was found to be a highly processive enzyme that operates preferentially on non-acetylated poly-mannuronan, though it was found to lack strict stereospecificity; it could cleave MM, MG and GG bonds yielding dimeric and trimeric products (67).

Although several studies have indicated that AlgX is essential for alginate production, until recently its exact role remained unclear. Robles-Price and colleagues (65) proposed that AlgX forms part of the periplasmic scaffold facilitating alginate translocation and secretion. Studies by Gutsche and colleagues (56) suggested that AlgX was also required for efficient polymerisation. The high sequence identity between AlgX and AlgJ, a protein involved in the O-acetylation of alginate, may represent a shared domain for alginate binding. Recently, the structure of AlgX was solved, and it was shown to have two domains: an N terminal SGNH hydrolase domain involved in the acetylation of alginate and a C-terminal carbohydrate-binding module which is thought to aid in alginate binding and orientation (24).

AlgK, another protein essential for successful translocation of alginate through the periplasm, has an unclear function. AlgK has multiple tetratricopeptide-like (TPRlike) repeats, a feature characteristic of proteins involved in the assembly of multiprotein complexes. This suggests that AlgK may play an important role in the assembly of functional alginate biosynthesis machinery. Keiski and colleagues (19) recently showed that AlgK is a lipoprotein associated with the outer membrane; moreover, it was shown that AlgK is required for localization of the porin AlgE to the outer membrane.

While the nascent alginate chain is transported across the periplasmic space, it can be modified by O-acetylation and epimerisation. Although the order of modification remains unknown, it is conceivable that O-acetylation precedes epimerisation, as O-acetylation blocks subsequent epimerisation or cleavage (Fig. 3). Although neither O-acetylation nor epimerisation are essential for alginate production, they can significantly alter the material properties of the resulting alginate (30). The O-acetylation of alginate is unique to bacterial alginates and significantly increased the water holding capacity of alginate; it is required for efficient biofilm development by Pseudomonads as well as protecting the organism from immune responses (35, 68).

During its transit through the periplasm, the nascent alginate is O-acetylated by the combined activities of AlgI, AlgJ and AlgF; however, these proteins are not essential for alginate production. AlgX has also recently been implicated in acetylation of alginate; the Ser-His-Asp catalytic triad found in the SGNH-like hydrolase domain was shown to be essential for acetylation (24). These proteins add O-acetyl ester linkages at the C2 or C3 position of M residues (Fig. 3) (23, 69). The source of the acetyl group is currently unknown; however, acetyl-coenzyme A is the likely candidate. The acetyl group is thought to be transported to the periplasm by AlgI, a cytoplasmic membrane protein with limited homologies to a *Bacillus subtilis* protein Ipa-4r (DltB), which transports an activated precursor during lipoteichoic acid biosynthesis (70). The second enzyme required for O-acetylation, AlgJ, is a periplasmic protein associated with the cytoplasmic membrane which shows high homology to AlgX; both proteins have sugar-binding-hydrolysing domains likely to be involved in substrate binding (65). Interestingly, *algI* and *algJ* are thought to be acquired by lateral gene transfer (69). AlgF is unique in that it does not have sequence homology to other proteins involved in O-acetylation. Because O-acetylation can restrict epimerisation and cleavage, being able to control the level of O-acetylation would allow some level of control over the extent of epimerisation and molecular weight.

Epimerisation of M residues to G residues leads to changes in alginate material properties. Generally, the presence of G residues in alginates allows for the formation of gels in the presence of divalent cations such as Ca^{2+} . The G residues must be found as consecutive stretches (designated 'G-blocks') in order to bind Ca^{2+} (Fig. 3).

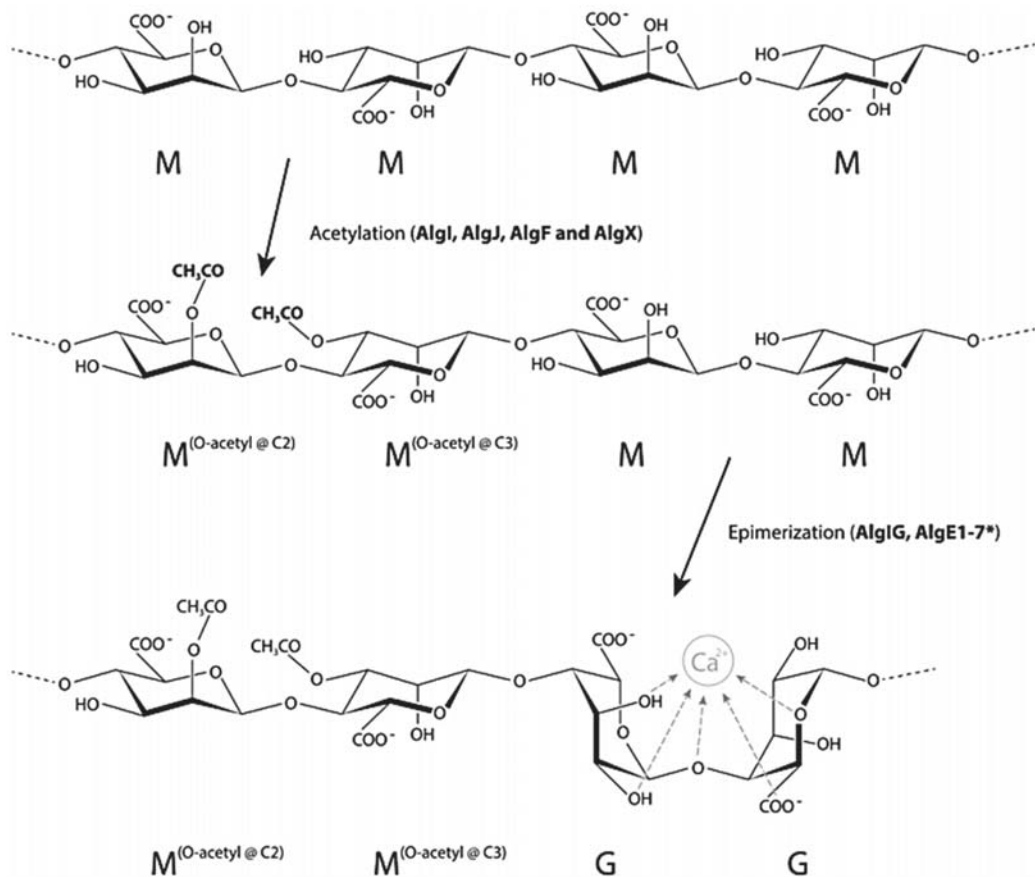


Fig. 3. Modification of bacterial alginate. Showing the acetylation of the first two M residues at C2 and C3 respectively; and the C5 epimerisation of the third and fourth M residues to G residues. The Ca^{2+} binding associated with G-blocks is shown. *AlgE1-7 are extracellular epimerases unique to *Azotobacter*.

The overall amount and length of these G blocks affect several properties of the gels, including stiffness, swelling and porosity (30). G-blocks also allow for interchain ion binding in an ‘egg-box’-like structure: the divalent cation interacts with two neighbouring G residues as well as with two G residues in a second chain generating interchain linkages. This is essential for the formation of hydrogels, with higher concentration of G blocks leading to stiffer gels. It is thought that MM or MG blocks are generally incapable of binding divalent cations in this way and are thought to act as elastic ‘hinges’ between the cross linked chains, though Donati and co-workers (71) have also demonstrated the ability of MG blocks to bind Ca^{2+} and to form gels in a similar manner.

AlgG is responsible for the periplasmic epimerisation of M to G residues. AlgG is a bifunctional protein which specifically catalyses the epimerisation of M residues to G

via protonation-deprotonation of C5 on the M residue in the alginate; it also forms an essential part of the periplasmic scaffold which protects the nascent alginate chain from AlgL-mediated degradation. Epimerisation is not essential for the production of high molecular weight (HMW) alginates; mutations disabling the catalytic residues of AlgG do not affect alginate yield (16, 72). The catalytic residues of AlgG reside in a shallow groove situated in a right-handed beta-helix fold, a common motif of carbohydrate-binding and sugar-hydrolysing proteins (21). The kinetics of this enzyme have been thoroughly examined, demonstrating that AlgG has higher affinity to larger substrates up to 20 residues (100 Å long) suggesting that several AlgG proteins may bind alginate simultaneously (73). While an apparent equilibrium of 75% G content is reached when AlgG is incubated with poly-M substrate *in vitro*, the G content of alginate produced by *P. aeruginosa* is significantly lower (typically less than 40%), suggesting that strict regulation and/or competition between modification pathways is occurring *in vivo* (74, 75). It should be noted that in addition to periplasmic AlgG, *A. vinelandii* also possess at least seven extracellular alginate epimerases, AlgE1-E7 with differing specificities and non-random epimerisation patterns (76).

Alginate secretion

The outer membrane beta barrel porin, AlgE, is responsible for the secretion of mature alginate (57). This protein is immunogenic and displays anion selectivity upon spontaneous incorporation into planar lipid bilayers (1, 77). Recently, the crystal structure of AlgE has been determined (62) and functional residues of the protein thoroughly probed (20). Despite the lack of sequence similarity, AlgE was found to be structurally similar to OprD, a substrate-specific nutrient uptake channel. The AlgE pore is lined with highly conserved, charged amino acid residues, in-part formed by the extracellular loops L3 and L7 folding into the lumen of the pore, which have been suggested to confer selectivity towards alginate and/or facilitate its efficient secretion across the outer membrane. AlgE has an unusually long and flexible periplasmic loop (L8) which appears to act as a 'stopper' in the deduced structure. It is thought that this region may interact with other subunits of the alginate biosynthesis machinery such as the TPR domains of AlgK and/or the membrane fusion domains of Alg44. It has been proposed that AlgK and AlgE interact, and this pair shares homology to enzymes involved in cellulose, Pel and poly-β-1,6-N-acetyl-D-glucosamine biosynthesis (19, 78).

Multiprotein alginate polymerisation/secretion complex

As mentioned above, it has long been suggested that the members of the alginate biosynthesis machinery form a multiprotein complex spanning from the IM, through the periplasmic space and into the outer membrane. Several studies have elucidated the specific protein–protein interactions involved in this complex (19, 20, 56, 79, 80). Recently, Rehman and colleagues (80) have undertaken a series of pull-down, cross-linking and mutual-stability experiments in an effort to map the specific protein–protein interactions in the multiprotein complex. This has led to a model for the alginate polymerisation/secretion complex as depicted in Fig. 4 (with experimentally deduced interactions indicated by triangles). Intriguingly, a key regulatory protein, MucD, appears to interact with the complex through AlgX. It is unclear what the function of this interaction is, but it has been suggested that MucD may be sequestered by an intact complex and becomes free to exert its regulatory role if the complex becomes unstable (56, 79).

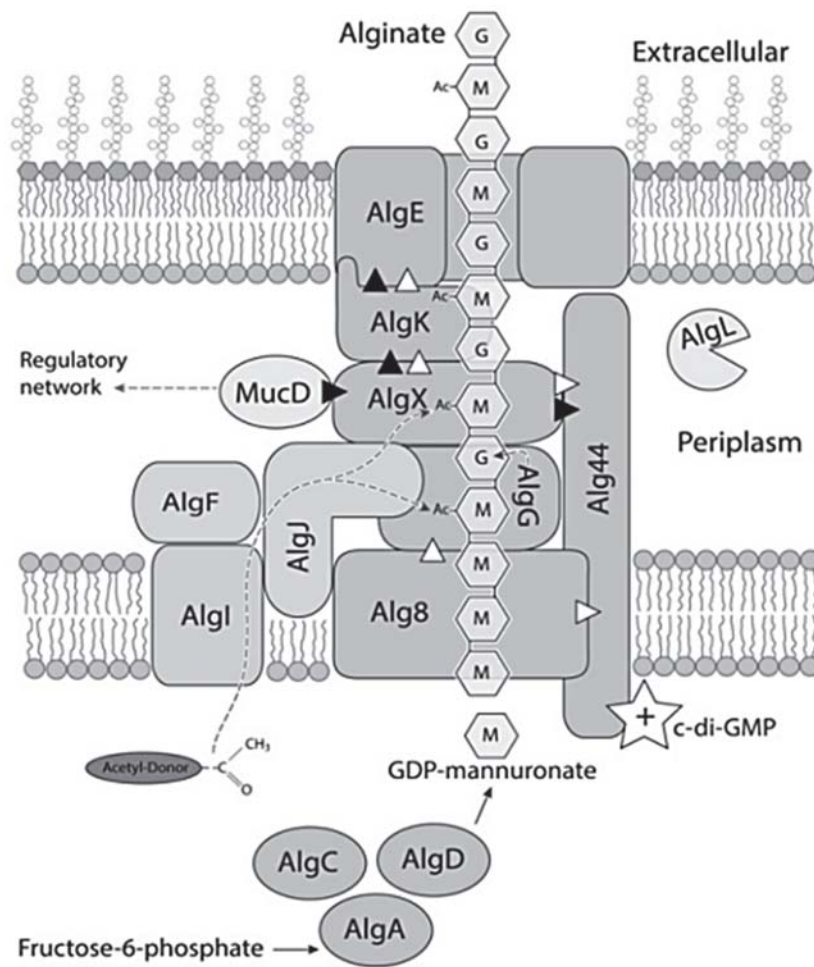


Fig. 4. Schematic representation of the alginate polymerisation/ secretion complex spanning from the inner membrane to the outer membrane. Acetylation by AlgI, AlgJ, AlgF and AlgX and epimerisation by AlgG are demonstrated by dashed lines. It remains unclear whether AlgJ or AlgX or both are responsible for the direct acetylation of the alginate chain. Deduced interactions are shown as triangles, with white triangles indicating a mutual stability relationship and black triangles representing a direct interaction as indicated by pull down data.

Regulation

The regulation of alginate biosynthesis is complex and involves transcriptional and post-translational levels of regulation, as well as several hypermutable regions of the genome in which mutant alleles lead to overproduction of alginate. Globally acting regulators as well as several alginate-specific regulators govern 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 (81).

Most of the genes involved in the genotypic switch are located within a single self-regulated operon (*algU*, *mucA*, *mucB*, *mucC* and *mucD*). This region is somewhat homologous to the well-characterized σ E region in *Escherichia coli*, containing the genes *rpoE* (encoding the σ E), *rseA*, *rseB*, *rseC*. AlgU is a key 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 (10, 82-84). AlgU is sequestered at the IM (and thus unable to bind RNA polymerase and initiate transcription) by the membrane anchored antisigma factor MucA (85-87). The periplasmic protein MucB binds to the periplasmic side of MucA and plays a negative regulatory role in alginate biosynthesis by protecting MucA from proteolysis. Release of AlgU and subsequent transcription appears to be initiated by a regulated intramembrane proteolytic (RIP) cascade leading to the degradation of MucA (88, 89). Several steps of the RIP cascade have recently come to light: The periplasmic protease AlgW (*E. coli* DegS homologue) initially cleaves MucA in response to envelope stress. Particular misfolded proteins (in particular, the outer membrane protein MucE) can bind to the PDZ activating domain of AlgW and cause its activation (de-repression). After cleavage by AlgW, MucA becomes susceptible to cleavage on the cytosolic side by the intramembrane protease MucP (*E. coli* RseP/ YaeL homologue) leading to the release of AlgU (90, 91) (Fig. 5). Three further cytosolic proteases, ClpX, ClpP1 and ClpP2, have recently been shown to be involved in the proteolysis of MucA (92). MucD is a periplasmic protease that appears to be playing a role antagonistic to that of AlgW. Disruption of *mucD* gene leads to a mucoid phenotype signifying a negative regulatory role. It is thought that MucD is involved in the degradation of misfolded proteins that would otherwise activate AlgW or MucP (88, 90, 91, 93). Although alginate production is the most apparent phenotype controlled by AlgU, it does not act exclusively on the alginate operon and has been shown to be involved in the transcriptional activation of genes with diverse functions, including genes involved in biosynthesis of other exopolysaccharides (83, 94, 95).

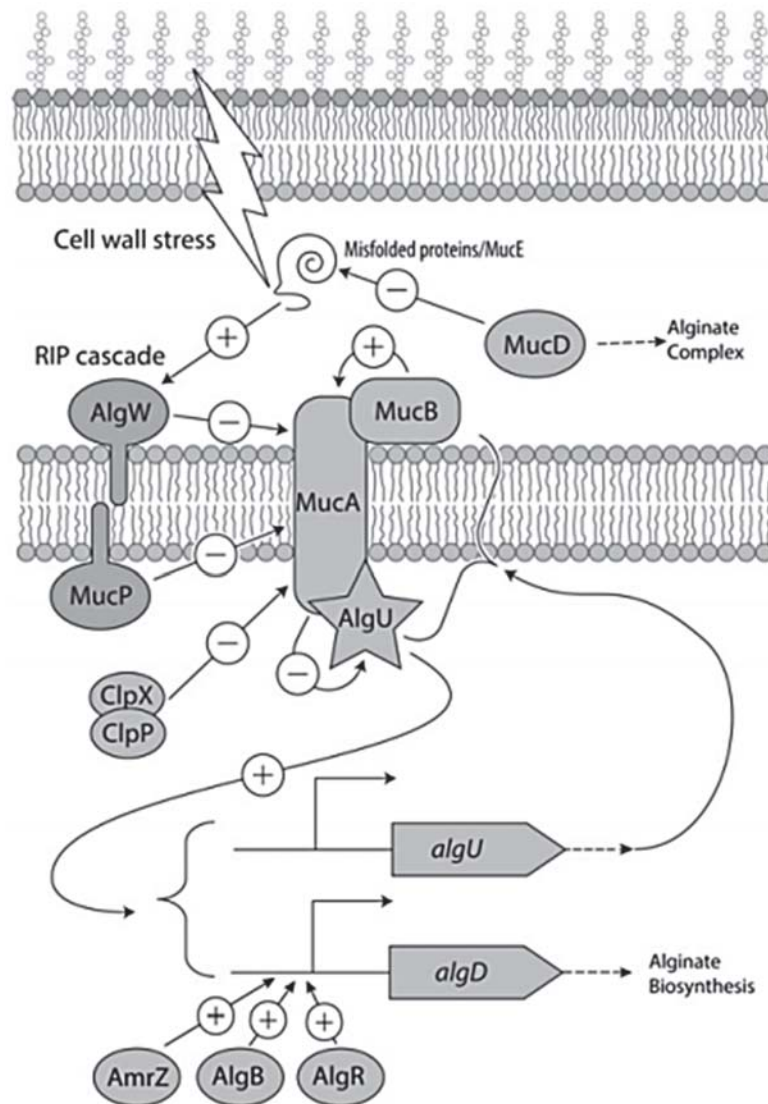


Fig. 5. Overview of the regulation of alginate biosynthesis. The periplasmic regulation and the regulated intramembrane proteolysis cascade are shown. The core alginate biosynthesis operon is indicated by the *algD* arrow. The 'genetic switch' operon is indicated by the *algU* arrow.

This operon has become known as the 'switch' locus because of the relatively high number of mutations found in this region in clinical mucoid isolates. The most common mutations occur in MucA and/or MucB, with up to 80% of mucoid *P. aeruginosa* clinical isolates containing mutations in the *mucA* gene. Most of these mutations result

in a premature stop codon and a truncated MucA rendering the RIP cascade redundant (96-100).

In addition to AlgU, several other proteins are required to initiate transcription of the alginate operon. This layer of regulation is known as ‘environmental stimuli’-based regulation. AlgR is a response regulator part of a two component regulator that binds to three sites in the *algD* promoter; the cognate sensory component of this regulatory pair is AlgZ (FimS) and strangely is not required for transcription of the alginate operon. AlgB is also part of a two-component regulator and binds to one site on the *algD* promoter; again, it’s activity is apparently independent of its cognate sensor kinase KinB (101). AmrZ (originally called AlgZ), an Arc-like DNA-binding protein, binds to one site on the *algD* promoter (102-104).

As mentioned above, the essential alginate biosynthesis protein Alg44 contains a c-di-GMP-binding/sensing PilZ domain in its C-terminus. This allows for an additional post-translational level of regulation (60). C-di-GMP is an important bacterial secondary messenger that has been linked to the posttranslational regulation of diverse processes such as motility, exopolysaccharide production and virulence (105). Recently, it has been demonstrated that one particular c-di-GMP-synthesizing protein, MucR, specifically influences the levels of alginate biosynthesis in *P. aeruginosa* presumably by generating a localized c-di-GMP pool in proximity to the alginate polymerisation/secretion multiprotein complex (15).

Applications of bacterial alginates

All current commercial alginates are isolated from farmed brown seaweeds, with over 30,000 metric tons produced annually (29). Its material properties, versatility and biocompatibility has led to alginates use as a viscosity regulator and stabilizer in foods, cosmetics and high-value medical applications including wound dressings, drug delivery systems and more recently in tissue encapsulation for regenerative therapy (106, 107).

While bulk alginate extracted from seaweed for the food and cosmetic industries is sold at prices as low as \$5 kg⁻¹, pharmaceutical grade alginates with defined MW, M/G ratios and hence more defined material properties cost more than \$100 g⁻¹ (Pronova web catalogue prices as of April 2013). The high-value applications of alginate in biotechnology and biomedical sciences require a steady supply of alginates with defined homogeneity in composition and material properties. Although seaweed alginates are

extensively used in biomedical applications as an immobilization material, it suffers from problems with mechanical stability, wide pore size distribution and osmotic swelling during physiological conditions. Furthermore, it is subject to heterogeneity in both ratio of G to M residues as well as molecular weight because of environmental and seasonal variation (29, 108, 109). This inability of algal alginate to fulfil specific demands created by biomedical industry can be overcome either by using bacterial alginate or using bacterial enzymes to modify algal alginates (78).

Because of the pathogenic nature of *P. aeruginosa*, any commercial bacterial production of alginate is likely to come from *A. vinelandii* or non-pathogenic *Pseudomonas* species. The M/G residue composition of *A. vinelandii* alginate is similar to those produced by seaweeds. By exploiting regulatory proteins discussed above, it is possible to engineer *A. vinelandii* strains with increased levels of transcription from the *algD* operon and thus increased levels of alginate production. Indeed, when this was combined with disruption of the polyhydroxybutyrate pathway (thus allowing more carbon sources for alginate biosynthesis) up to 7 g l⁻¹ of alginate was obtained (110, 111). Furthermore, disruption of the Na⁺-translocating NADH: ubiquinone oxidoreductase complex in *A. vinelandii* leads to an alginate overproducing phenotype, this alginate also had a higher degree of acetylation and a lower G/M ratio, though the exact mechanism remains unclear (112, 113).

Viscosity is also influenced by the molecular weight of alginate. HMW alginate (more viscous) can be produced by *A. vinelandii* when the dissolved oxygen is controlled and/or the *algL* gene is disrupted (114-117). Recently, a study has linked the increased expression of alginate polymerase *alg8/alg44* with the production of HMW alginate in *A. vinelandii* (114). Interestingly, a mutant of *A. vinelandii*, with increased expression of the *algD* operon and disruption of polyhydroxybutyrate biosynthesis, produced alginate with an extremely HMW (4000 kDa) (111).

As mentioned above, *A. vinelandii* secretes seven C-5 epimerases each introducing a specific ratio and pattern of G residues. For example, AlgE2 and AlgE6 introduces continuous stretches of G-residues forming G-blocks while AlgE4 actions results in the formation of MG blocks (76). Utilizing these enzymes allows a tighter control of the material properties of alginate. Indeed, *A. vinelandii* epimerases have been employed to modify alginate to exhibit material properties required for immobilization of living cells (108, 118). A mutant of *P. fluorescens* lacking the epimerase AlgG can be used to produce poly-M (72). High M content alginates are of particular practical interest for

some types of cell transplantations because of their particular material properties and biocompatibility (31, 119); poly-M hydrolysis products are known to exert anti-inflammatory activity (120).

As mentioned above, a key difference between algal and bacterial alginates is that the latter is O-acetylated. A higher degree of O-acetylation significantly increases viscosity and pseudoplastic rheology (30, 121). The level of O-acetylation can be controlled reasonably well using specific strains/mutants or altering the growth media and controlling cultivation conditions such as aeration, pH and temperature (112, 122, 123). In addition, live immobilized *P. syringae* cells have been successfully used to acetylate seaweed-derived alginates (124).

Understanding and harnessing these mechanisms of alginate production and modification in bacteria could enable manufacture of tailor made bacterial alginates for high value medical and biotechnological applications.

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Chapter III

Genetics and Regulation of Bacterial Alginate Production

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Abstract

A vast range of extracellular polysaccharides are produced by bacteria in order to adapt to and thrive in diverse environmental niches. Many of these polymers have attracted great attention due to their implication in biofilm formation, capsule formation, virulence, or for their potential medical and industrial uses. One important exopolysaccharide, alginate, is produced by various *Pseudomonas* spp. and *Azotobacter vinelandii*. Alginate is of particular interest due to its role in the pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. Here, we will discuss the genetic organization and distribution of the genes involved in the biosynthesis of this significant polymer. The complex regulatory networks involved in the production of bacterial alginate will be reviewed, including transcriptional, posttranscriptional and posttranslational forms of regulation.

Introduction

Bacterial alginates have attracted great attention due to their role in the pathogenesis of the opportunistic human pathogen *Pseudomonas aeruginosa* and several phytopathogenic *Pseudomonas* spp. Furthermore, due to the use of seaweed-derived alginates in the food and pharmaceutical industries, the potential of a bacterial source for the production of more homogenous alginates (primarily from *Azotobacter vinelandii*) has been investigated (78). Alginate is an anionic linear polymer composed of β -1,4-linked mannuronic acids and its epimer, α -L-guluronic acid. The molecular mechanisms involved in alginate biosynthesis have attracted a great deal of research over the past few decades, but they are still not fully understood.

The mechanisms behind alginate biosynthesis are very similar among *Pseudomonas* spp. and *A. vinelandii*, although the physical role and properties of the resulting polymers differ substantially. Its production is perceived to be an important virulence factor for plant and human pathogenic *Pseudomonas* species (125). Indeed, the conversion of the opportunistic human pathogen *P. aeruginosa* from a non-mucoid phenotype to an alginate-overproducing mucoid phenotype early after the infection of cystic fibrosis patients is associated with a decline of pulmonary function and survival rate (2).

Initially thought to be the key component of *Pseudomonas* biofilms, the production of alginate was shown to be not essential for biofilm formation (126). Nevertheless, studies have shown that it does play a role in the maturation of biofilms and the formation of thick and highly structured biofilms with differentiated microcolonies (35, 36). It has been suggested that alginate functions to maintain hydration of the cells and is required for survival and biofilm formation under desiccating conditions. Alginate was shown to be integral to biofilm architecture under water-limiting conditions but does not play a significant role under fully hydrated conditions. Furthermore, it was shown that alginate production is induced under desiccating conditions (127, 128). This appears to correlate with the finding that *P. aeruginosa* clinical isolates from the cystic fibrosis lung environment, which displays airway surface liquid dehydration, typically possess a mucoid, alginate-overproducing phenotype (97, 129-131).

The production of thick biofilms containing alginate has been shown to impede the diffusion of various antimicrobial agents and can protect the bacteria from common bactericides used in plants (4, 132-134). Encased cells are also protected from host

defence mechanisms such as interferon- γ -mediated macrophage killing (135) and other antibody-independent opsonic killing mechanisms (68, 136). Furthermore, alginate can scavenge reactive oxygen species, such as superoxide radicals and hypochlorite, which are used by macrophages and neutrophils for pathogen killing and also released during the hypersensitive response plant defence system (5, 137). It should be noted that at least two other exopolysaccharides, Psl and Pel, are involved in *P. aeruginosa* biofilm formation (138).

The soil-dwelling *A. vinelandii* is unique among the Azotobacteriaceae in that it poses a characteristic life cycle, which includes the formation of a dormant desiccation-resistant cyst. The formation of this cyst requires alginate production, and the tough extracellular coating is composed of a high concentration of alginate (139). The outermost alginate layer (exine) has a high content of consecutive guluronic acid residues, which results in a more rigid alginate. This phenotype is caused by the secretion of several extracellular alginate epimerases, unique to *A. vinelandii*, which extensively modify the ratio of manuronic acid and guluronic acid block residues, and thus the material properties of the secreted alginate (140, 141). It should be noted that *A. vinelandii* can also show a mucoid colony phenotype and secrete alginate during vegetative growth. This may indicate that there are different regulatory signals and pathways involved in the production of alginate in *A. vinelandii*. The biochemical steps of alginate biosynthesis from precursor synthesis, polymerisation, periplasmic transit, modification and secretion have been covered in detail in several recent reviews (142, 143). Here, we will discuss the genetics and regulation of the bacterial alginate biosynthesis in detail.

Genetics

The core alginate biosynthesis and regulatory genes are widely distributed throughout the *Pseudomonas* rRNA homology group I. The biosynthesis of alginate has been demonstrated in several members of this group: *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. syringae*, *P. mendocina* and *A. vinelandii* (144, 145). It should be noted here that recent bioinformatic analyses of the *A. vinelandii* genome suggested that it might be better reclassified as a *Pseudomonas* species (146-148). The genes involved in alginate biosynthesis and regulation are virtually identical between these *Pseudomonas* spp. and *A. vinelandii*, although their regulation is slightly different. All but one of the core genes involved in alginate biosynthesis are contained within a single 12-gene operon initially described by Chitnis and Ohman (10): *algD*, *alg8*, *alg44*, *algK*, *algE* (named *algJ* in *A. vinelandii*), *algG*, *algX*, *algL*, *algI*, *algJ* (named *algV* in *A. vinelandii*), *algF* and *algA*.

In *P. aeruginosa*, this cluster is primarily transcribed from a single AlgU (σ^{22})-dependent promoter located upstream of *algD* (38). In contrast, the *A. vinelandii* alginate gene cluster has at least two promoters upstream of *algD*, one AlgU-dependent and one RpoS (σ^s)-dependent (149). *Azotobacter vinelandii* also has three additional internal promoters, one σ^s type promoter upstream of *alg8*, one upstream of *algG* (unknown control) and one upstream of *algA* (also unknown control) (40, 139, 150, 151). Recently, evidence supporting internal promoters (upstream of *algG* and *algI*) in the *P. aeruginosa* alginate operon was also presented (41). The fact that several of these promoters are upstream of the genes responsible for polymer modification (acetylation and epimerisation) may suggest that these internal promoters are involved in regulating the level or polymer modification.

Regulation of alginate biosynthesis

Bacterial alginate biosynthesis is controlled by a complex regulatory network. Here, we describe how bacterial alginate production is regulated. Due to its clinical significance, most of our information comes from studies in *P. aeruginosa*. Where differences between species are apparent, they will be discussed.

Regulated intramembrane proteolysis (RIP) of the MucA anti-sigma factor

The master regulator of alginate biosynthesis is the alternate sigma factor AlgU (previously called AlgT or σ^{22}), a homologue of the stress response regulator RpoE from *E. coli*. AlgU is classified as an extra cytoplasmic function (ECF) sigma factor, a family of sigma factors that confer resistance to envelope stress caused by antimicrobial and oxidizing agents, elevated temperatures, and osmotic imbalances. Under uninduced conditions the activity of AlgU is sequestered (Fig. 1A), various environmental cues can lead to the release of AlgU, allowing activation of AlgU-dependent promoters (Fig. 1B). AlgU is encoded in an operon containing four other genes, *mucA*, *mucB*, *mucC* and *mucD*, the products of which modulate its activity. This operon is known as the switch locus for alginate biosynthesis because mutations in this region are frequent among clinical alginate-overproducing strains of *P. aeruginosa*. The majority of these mutations inactivate the anti-sigma factor MucA, forcing the system into a permanent ‘on’ state (98, 152) (Fig. 1C).

Like other ECF sigma factors (such as RpoE), AlgU promotes the expression of its own operon, along with several other genes involved in alginate biosynthesis and regulation:

algR, *algB*, *algD*, *algC* and *amrZ* (94, 153, 154). In addition, AlgU has been determined to be involved in the regulation of motility, quorum sensing and virulence (7). MucA and MucB are the functional equivalents of RseA and RseB in *E. coli* (85). Like RseA and RseB, which negatively regulate RpoE, MucA and MucB repress AlgU. MucA is a transmembrane protein that sequesters AlgU at the inner membrane, while MucB binds to the periplasmic domain of MucA, protecting it from proteolysis (89, 93). MucC is an inner membrane-associated protein with an unclear role. MucD is a periplasmic protease/chaperone generally considered to have a negative regulatory role (88-90, 155, 156). Within the *mucC*, ORF is an alternative promoter region for *mucD* expression (156).

In response to envelope stress, the ECF sigma factors AlgU and RpoE are released from their anti-sigma factor complexes (MucA-MucB and RseA-RseB, respectively) through a well-conserved signal transduction pathway known as a RIP cascade involving several proteases (Fig. 1B). These proteases are divided into three classes: site-1, site-2 and cytoplasmic proteases. The site-1 protease AlgW in *P. aeruginosa* and DegS in *E. coli* initiate the RIP cascade by degrading the periplasmic domain of the anti-sigma factor MucA/RseA primarily at 'site-1' (90, 157). Site-1 cleavage is the rate limiting step and is ultimately required for subsequent cleavage. This is followed by intramembrane proteolysis of MucA/RseA by the site-2 protease, MucP in *P. aeruginosa* and RseP in *E. coli* (90, 158-160). After site-2 cleavage, the truncated MucA/RseA, still bound to AlgU/RpoE, is released into the cytosol where it is rapidly degraded by ATP-dependent cytoplasmic proteases, freeing the sigma factor (AlgU/RpoE) and allowing it to interact with RNA polymerase to drive expression of its regulon, including the alginate operon (92, 161).

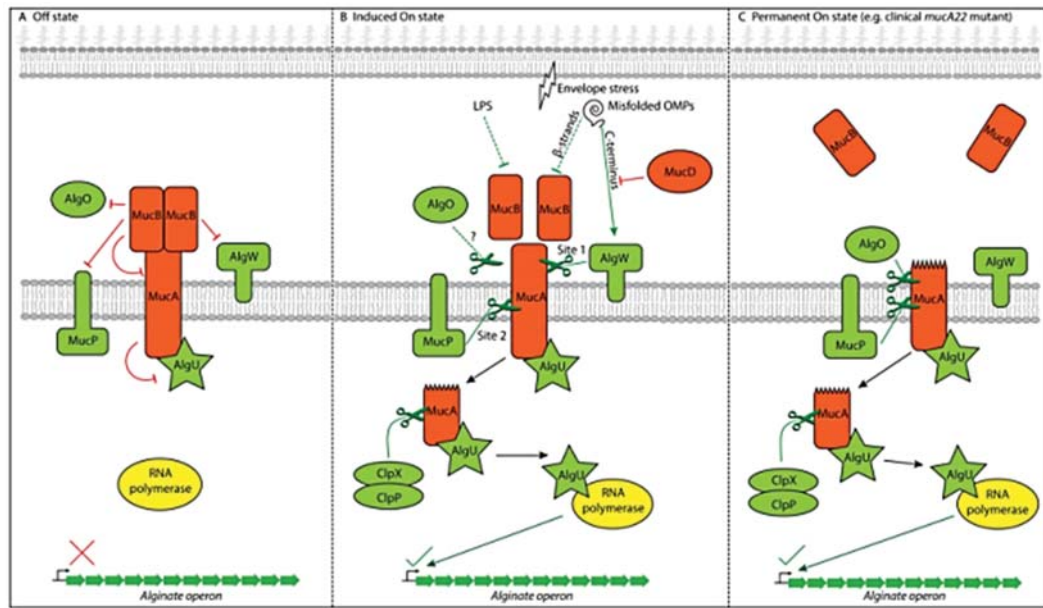


Fig. 1. MucA RIP cascade (Attention readers: please refer to the online full colour version). Green proteins and lines have a positive effect on alginate production; red proteins have a negative effect on alginate production. Dotted lines indicate an unknown/unclear mechanism. (A) In the absence of envelope stress (thunderbolt), the system is turned OFF. AlgU is sequestered at the inner membrane by its cognate anti-sigma factor complex MucA–MucB. The MucA–MucB interaction protects MucA from proteolysis by the site-1 and site-2 proteases AlgW and MucP. (B) Under conditions of envelope stress, the system is turned ON. Envelope stress damages OM components, triggering the RIP cascade. Misfolded OMPs activate AlgW. The protective MucA–MucB interaction is destabilized by the beta strand motifs of OMPs as well as mislocalized lipopolysaccharides. MucD can degrade and/or repair damaged outer membrane proteins that activate AlgW. Cleavage of MucA by AlgW is followed by site-2 proteolysis by MucP, releasing the cytoplasmic domain of MucA with AlgU bound into the cytosol where ClpXP degrade MucA, freeing AlgU to interact with RNA polymerase and drive expression of alginate production genes. (C) Example of a clinical *mucA* mutant (*mucA22*) with a truncated MucA that can no longer interact with MucB, rendering it susceptible to proteolysis by AlgO and possibly other proteases.

Studies in *P. aeruginosa* and *E. coli* have shown that under uninduced (unstressed) conditions, AlgW/DegS do not have access to the initial cleavage site of MucA/RseA and thus the RIP cascade cannot be initiated (Fig. 1A). MucB/RseB is bound to the periplasmic side of MucA/ RseA and protects ‘site-1’ from cleavage by AlgW/DegS (90, 162-164) (Fig. 1B).

Envelope stress detrimentally affects trafficking, localization and stability of outer membrane proteins (OMP) and lipopolysaccharides (LPS), leading to an accumulation

of misfolded and/or mislocalized OM components in the periplasm (165). Some of these components serve as signals to activate the RIP cascade (Fig. 1B). For instance, misfolded OMPs with conserved carboxyl-terminal amino acids (e.g. MucE from *P. aeruginosa* and OmpX from *E. coli*) bind to the PDZ domains of AlgW/DegS, derepressing these proteases (90, 93, 163, 166). Meanwhile, mislocalized LPS relieves MucB/RseB inhibition by disrupting its interaction with MucA/RseA, rendering the antisigma factors susceptible to proteolysis (160, 165). It is also suggested that the RseB–RseA (and possibly MucB–MucA) interaction is destabilized by lipophilic beta strand motifs of OMPs (160, 167). The requirement of a dual signal allows rapid response to serious membrane disturbances while preventing overreaction to minor issues, affecting only a subset of OM components (Fig. 1B).

After cleavage by AlgW/DegS, the anti-sigma factors are further degraded within or near the cytoplasmic membrane by the site-2 protease MucP/RseP. These proteases have well-conserved HExxH and LDG motifs typical of zinc metalloproteases (90, 157, 168, 169). In contrast to the site-1 proteases, the two PDZ domains of RseP (and possibly of MucP) are not involved in sensing envelope stress (164, 170). Rather, these PDZ domains serve as a switch; they activate proteolytic activity in the presence of their substrate (truncated MucA) (171, 172). In *E. coli*, proteolysis of RseA by RseP is inhibited by the periplasmic glutamine-rich domain of RseA. This region is removed by the action of the site-1 protease allowing RseP to cleave RseA (168, 169).

After cleavage by the site-2 proteases, the N-terminal cytoplasmic portion of MucA/RseA bound to AlgU/RpoE is released into the cytosol where it is degraded, in *P. aeruginosa* predominately by the cytosolic protease complex ClpXP (92) (Fig. 1B). In *E. coli*, several other generic proteases – ClpAP, Lon, FstH and HslUV – also take part in this final degradation step (164), which could indicate that other cytosolic proteases are involved in the proteolysis of MucA in *Pseudomonas*. Given that the binding affinity between RseA and RpoE is several orders of magnitude greater than the interaction between RpoE and core RNA polymerase, it is apparent that this degradation of cytoplasmic RseA is essential for activation of RpoE (173).

In addition to site-1, site-2 and cytoplasmic proteases (AlgW, MucP and ClpXP) described above, several other proteases that participate in or help modulate the RIP cascade have been identified. MucD, a periplasmic serine protease and chaperone-like protein, negatively regulates the RIP cascade by chaperoning and/or degrading misfolded OMPs or other proteins that would otherwise activate AlgW and potentially

MucP (90, 91, 174) (Fig. 1B). Studies have shown that the *E. coli* homologue of MucD, DegP, also degrades and refolds damaged proteins (175). It is conceivable that during mild envelope stress, MucD/DegP forms the first line of defence by repairing or removing damaged OMPs. However, when the accumulation of misfolded proteins exceeds the capacity of MucD/DegP, site-1 proteases are activated, triggering the RIP cascade and activating the RpoE/AlgU dependent stress response system to restore homeostasis. To complicate this situation, MucD appears to interact directly with a member of the core alginate biosynthetic machinery (AlgX) (56, 79). Hay and colleagues (79) proposed that MucD could be sequestered by intact machinery and is subsequently released upon its disassembly or destabilization.

A different pathway exists in clinical *P. aeruginosa* strains, which show mutations in *mucA* (176). These strains typically produce a version of MucA highly susceptible to proteolysis due to a truncation at their periplasmic C-terminus preventing interaction with MucB (Fig. 1C). Through screening for spontaneous suppressor mutations in these constitutive mucoid strains, an additional periplasmic protease, AlgO (PA3257/Prc), with an apparent positive regulatory role was identified. In these mutants, the protease AlgO is apparently required for degradation of truncated MucA (176, 177). However, AlgO is not required for alginate production in strains harbouring wild-type MucA (90). If and how it is directly involved in the WT MucA RIP cascade remains unknown; however, it is apparent that the *E. coli* homologue, Prc, does not appear to act in the RseA RIP cascade.

Interestingly, at least one of these periplasmic proteases, MucP, is also involved in the regulation of FpvR, an anti-sigma factor, freeing the sigma factors PvdS and FpvI, which regulate genes involved in the synthesis and secretion of several siderophores in *P. aeruginosa* (178). So far, it is unknown whether AlgW or MucD also participates in other RIP cascades.

One key question that remains unanswered is what are the specific 'real-life' environmental cues that lead to this RIP cascade being activated. Most of the steps discussed above have been deduced through the use of artificial membrane stress (e.g. growth in the presence of D-cycloserine, ammonium metavanadate and/or triclosan), or the mutation or overexpression of regulators (e.g. MucA, MucD or MucE); how this is related to an *in vivo* system remains unclear. The fact that many clinical isolates contain mutations in *mucA* or *mucB* suggests that this system may become redundant in *in vivo* chronic infections, but it might play a role in the early adaptation to the host

environment, perhaps in response to envelope stresses associated with desiccating environments (127). Indeed, it has been demonstrated that non-mucoid strains can be induced to produce alginate when exposed to a mouse lung infection model (179).

Transcriptional regulation

Alginate production is regulated transcriptionally by various sigma factors, two component signal transduction systems and other DNA binding proteins (Fig. 2A–C). As discussed above, the availability of the essential alginate sigma factor, AlgU (σ^{22}/σ^E), is controlled by the complex RIP cascade. Once AlgU is released, several other steps take place to allow it to bind to the RNA polymerase and activate the *algD* promoter. During alginate production, the general housekeeping sigma factor RpoD (σ^{70}) is sequestered by the anti-sigma factor, AlgQ (or potentially other anti- σ^{70} factors), thereby blocking its interaction with RNA polymerase and allowing the alternative sigma factors AlgU to bind to RNA polymerase and mediating transcription of the alginate biosynthesis operon (180-182).

An additional interesting sigma factor antagonism also takes place with the RpoN (σ^{54}) alternate sigma factor. The *algD* promoter contains overlapping recognition sites for both AlgU and RpoN. It has been proposed that RpoN binds to the *algD* promoter under particular conditions (i.e. in a nitrogen rich environment), and acts as a repressor blocking the binding of the AlgU-RNA polymerase holoenzyme and inhibiting transcription (183). To complicate this situation, in particular, mucoid mutant strains (*muc23/PAO579*) alginate production is not dependent on AlgU, and *algD* transcription is driven by RpoN (Fig. 2D).

Transcription from the *algD* promoter is regulated by a range of other DNA-binding proteins, which promote *algD* operon expression by latching onto its promoter region (103, 184) (Fig. 2B and C). AmrZ (previously called AlgZ) is a ribbon-helix-helix DNA-binding protein, which is required for efficient transcription from the *algD* promoter. The versatility of AmrZ's DNA-binding domain allows it to repress or activate expression of various genes. As well as activating transcription of the *algD* operon, AmrZ represses the expression of its own gene, negatively regulates Psl exopolysaccharide biosynthesis, is involved in the control of flagella production, and is generally considered to be a positive regulator of virulence mediating the transition of *P. aeruginosa* biofilm infections from colonizing to chronic forms (84, 153, 185-188).

AlgQ (previously known as AlgR2) is a DNA-binding protein that has been shown to be a positive regulator of alginate biosynthesis and various other phenotypes; it is suggested to be a global transcriptional regulator in *P. aeruginosa* (189). The precise mechanism by which AlgQ regulates transcription of *algD* is not known, although it has recently been suggested that the positive regulatory role observed may be indirect: AlgQ can also bind to the housekeeping sigma factor RpoD, making it unavailable for binding to the RNA polymerase complex, and it is possible that this step promotes *algD* transcription (as well as other alternative sigma factor-dependent promoters) by making the RNA polymerase complex available for AlgU binding (182, 190, 191) (Fig. 2B and D).

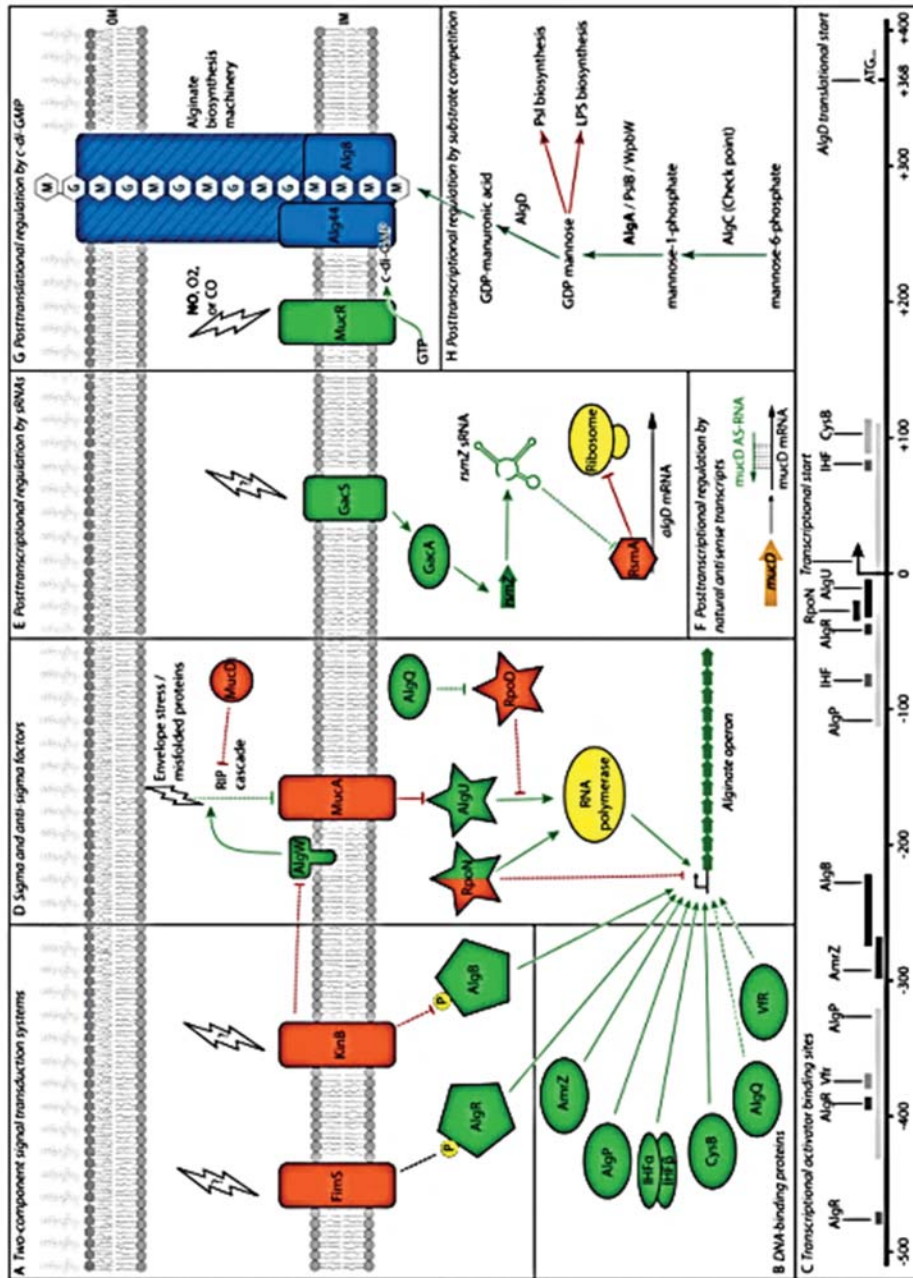


Fig 2. Schematic representation of various regulatory mechanisms of alginate biosynthesis. (Attention readers: please refer to the online full colour version) Green and red proteins/lines promote and suppress alginate production respectively. Dotted lines indicate an unknown/unclear mechanism. (A) Two-component signal transduction systems FimS/AlgR and KinB/AlgB. (B) Transcriptional regulation through DNA-binding proteins. (C) Schematic map of the approximate binding sites of various transcriptional regulators on the *P. aeruginosa algD* promoter. (D) Sigma/anti-sigma factors. (E) Posttranscriptional regulation through the Gac/Rsm sRNA system in *Azotobacter vinelandii*. (F) Posttranscriptional regulation through a natural antisense transcript (MucD-AS) that promotes alginate production by blocking the translation of *mucD* mRNA. (G) Posttranslational regulation by c-di-GMP. MucR synthesizes a pool of c-di-GMP near the alginate co-polymerase Alg44. Binding of c-di-GMP to Alg44 is essential for alginate biosynthesis. (H) Posttranscriptional regulation by substrate competition.

AlgP (previously called HpI and AlgR3) is a histone-like DNA-binding protein that has been demonstrated to positively regulate transcription from the *algD* operon in response to signals such as nitrogen availability (192, 193). An integration host factor like heterodimer (IHF α and IHF β) has also been shown to bind to the *algD* promoter and is required for efficient *algD* transcription (194). CysB, a transcriptional factor of the LysR superfamily, is also involved in binding to and activating transcription from the *algD* promoter (195) (Fig. 2B and C).

Another DNA-binding protein, Vfr, has been implicated in transcription from the *algD* promoter, although the evidence is somewhat indirect. Vfr is a homologue to the *E. coli* DNA-binding protein CPR (cAMP receptor protein). This protein is involved in catabolite repression, and it was shown that the *algD* promoter was moderately sensitive to glucose repression in *P. aeruginosa*. Furthermore, the *E. coli* CRP-cAMP complex bound the *algD* promoter and activated its transcription (196). Vfr can bind cAMP; however, since glucose does not apparently alter cAMP level in *P. aeruginosa* and that Vfr is not required for catabolite repression control in *P. aeruginosa*, the role of catabolite repression in this signalling is unclear (197) (Fig. 2B and C).

The KinB-AlgB and FimS (previously called AlgZ)-AlgR two-component signal transduction systems also control the expression of alginate production genes (Fig. 2A). Signal transduction in this class of proteins occurs upon stimulation by an environmental cue. Generally, the sensor kinase protein is autophosphorylated and subsequently facilitates the activation of the response regulator via phosphotransfer. Currently, the exact environmental cues of KinB-AlgB and FimS-AlgR systems remain unknown. Both the response regulators, AlgB and AlgR, bind to the *algD* promoter, activating the expression of alginate biosynthesis genes (198). However, recent findings suggest that these two component signal transduction systems act in a noncanonical manner (at least for KinB-AlgB). The *algD* activating function of AlgB and AlgR does not appear to be dependent on their cognate sensor proteins KinB and FimS, and furthermore their phosphorylation is also not required (101, 199). It has been suggested for the KinB-AlgB system that KinB may possess a phosphatase function, and that its primary role may not be to phosphorylate AlgB but to dephosphorylate it in response to environmental cues (200) (Fig. 2A). This may allow KinB-AlgB to act as global regulator controlling the switch from an acute virulence phenotype (motile, pyocyanin and elastase-producing) with AlgB un-phosphorylated, to a chronic virulence phenotype (non-motile, alginate-producing) with AlgB phosphorylated. Furthermore, the

phosphorylation of AlgB is not dependent on KinB and its phosphorylation may be via unknown alternative sensor kinases (200, 201). To further complicate this situation, it has recently been shown that KinB-AlgB may play a role in the AlgW-mediated degradation of the MucA anti-sigma factor, although the mechanisms remain unclear (202) (Fig. 2A and D). Similar to the results observed with KinB-AlgB mutants, analysis of mutants of the FimS-AlgR system seems to suggest that it is not regulating alginate biosynthesis in a canonical sensor kinase – response regulator manner: disruption of AlgR suppresses alginate production suggesting a positive regulatory role, whereas disruption of FimS increases alginate production suggesting a negative regulatory role (203) (Fig. 2A). The AlgR response regulator regulates various other important virulence factors, including LPS, hydrogen cyanide production, and twitching and swarming motility (204-207). It should be noted that although *A. vinelandii* possesses a copy of the *algR* gene with high homology to the *P. aeruginosa* *algR* gene, it does not appear to be required for alginate biosynthesis or transcription of *algD* (although it is essential for cyst formation). Also, *A. vinelandii* does not have a *fimS* homologue upstream of *algR*, as can be found in *P. aeruginosa* (6).

With so many DNA-binding proteins regulating *algD* operon expression (Fig. 2C), it is likely that the quaternary structure of the *algD* promoter region significantly affects transcription rate. Hence, various combinations of regulatory elements binding to the *algD* promoter and other alginate-related promoters would undoubtedly result in varying levels of gene expression and alginate yield. Worth noting is the plethora of other pathways controlled by the above-mentioned transcription regulators – this includes but is not limited to quorum sensing, biofilm formation and motility, and metabolite synthesis and nutrient acquisition (91, 104, 181, 208).

Posttranscriptional regulation

There is an emerging role of noncoding small RNAs (sRNA) in the regulation of alginate, at least in *A. vinelandii* (Fig. 2E). The GacS-GacA two-component system is known as a regulator of several unrelated pathways, including biofilm formation, quorum sensing, oxidative stress, central carbon metabolism, virulence and biofilm formation in various bacterial species. The response regulator GacA activates the transcription of several genes specifying noncoding sRNAs (209, 210). In *Pseudomonas* spp., these sRNAs have been classified into three families: RsmZ, RsmY and RsmX (211). These sRNAs work by binding to a translational regulatory protein, RsmA, which

would otherwise bind to and repress translation of various mRNAs (212). In *A. vinelandii*, it was shown that RsmA binds to the 5' leader of the *algD* mRNA transcripts and represses translation (213). In *A. vinelandii* as well as various *Pseudomonas* spp., disruption of the GacS-GacA two-component system leads to reduction in alginate production or *algD* transcription (149, 213-216). Alginate production can be restored in *A. vinelandii* through the constitutive expression of several sRNAs (RsmZ1 and RsmZ2) (213). Furthermore, in *P. aeruginosa*, Burrowes and colleagues (217) analysed the transcriptome of an *rsmA* mutant and found that *algD* and *alg8* were upregulated. Also, a recent screen of mutants incapable of inducible alginate production identified that *gacA* is required for inducible alginate production (218). It is not known whether the Rsm system directly controls *algD* expression in *Pseudomonas* spp. as it does in *A. vinelandii*.

Recently, Yang and colleagues (219) identified a natural antisense transcript (*mucD*-AS) that when overexpressed significantly induced alginate production and biofilm formation in *P. aeruginosa*. This natural antisense transcript is transcribed from the opposite strand of *mucD* gene locus and appears to block MucD production (Fig. 2F). If and how this mechanism is significant in the natural settings remains unknown.

Posttranslational regulation

Alginate biosynthesis is also regulated posttranslationally by bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) binding to the PilZ domain of Alg44, the putative co-polymerase of the alginate biosynthesis machinery (15, 60) (Fig. 2G). c-diGMP is a generic secondary messenger molecule utilized by bacteria for regulation of motility, exopolysaccharide production and virulence (220).

The inner membrane protein Alg44 has been implicated in the posttranslational regulation of alginate biosynthesis in response to c-di-GMP. Alg44 is a core member of the alginate polymerisation-secretion complex and is essential for alginate polymerisation. Alg44 protein is a multi-domain protein consists of a cytoplasmic c-di-GMP binding/sensing PilZ domain, a transmembrane region leading to a periplasmic domain with similarities to membrane fusion proteins such as MexA, a membranebridging protein involved in the multidrug efflux system of *P. aeruginosa* (58, 221). Recent mutual stability studies suggested that Alg44 is part of alginate polymerisation/secretion multiprotein complex, with its stability impacted by the outer membrane secretion pore AlgE (57, 77, 80). Although the exact role of Alg44 in

alginate polymerisation is not understood, it is known that the PilZ domain of Alg44 is essential for alginate biosynthesis (60). The mechanism by which the binding of c-di-GMP by Alg44 is translated to alginate biosynthesis is unknown. Several recent studies, investigating the effect that the binding of c-di-GMP to PilZ domains has on protein structure, have shown that binding induces various conformational changes within the PilZ domain, which may expose surfaces involved in protein–protein interactions (222–224). Similar c-di-GMP-dependent polymerisation processes have been observed in the biosynthesis of the Pel polysaccharide in *P. aeruginosa* (225), cellulose in *Gluconacetobacter xylinus* and *Rhodobacter sphaeroides* (61, 226), and poly- β -1,6-Nacetylglucosamine (poly-GlcNAc) in *E. coli* (227). In the latter case, binding of c-di-GMP stabilized the interaction of two inner membrane proteins involved in the polymerisation and promoted their enzymatic activity. Perhaps the most relevant information to date with respect to the mechanisms behind the role of c-di-GMPs in alginate biosynthesis is derived from analysis of the cellulose synthase complex BcsA–BcsB in *R. sphaeroides*, which demonstrated that binding of c-di-GMP to the PilZ domain of BcsA induces a conformational change allowing sugar nucleotide precursor, UDP-glucose, to access its glycosyltransferase catalytic site (226). Mutual stability experiments point to an interaction between Alg44 and the glycosyltransferase, Alg8, and it has been proposed that these two proteins form an inner membrane alginate polymerase/co-polymerase complex (80). Both BcsA and Alg8 are categorized as family II glycosyltransferases (228).

Hay and colleagues (15) identified the inner membrane protein MucR, which presumably produces a localized pool of c-di-GMP in close proximity to Alg44. This protein has a transmembrane domain separating a periplasmic N-terminus, harbouring MHYT domain with putative di-atomic gas sensing function (O_2 or NO has been proposed to be the signal), and a cytoplasmic C-terminus bearing GGDEF and EAL motifs required for c-di-GMP synthesis and degradation respectively (15, 229). Li and colleagues (230) have demonstrated that the c-di-GMP synthesizing and degrading activities of MucR were growth mode-dependent; it synthesized c-di-GMP during biofilm but degraded it during planktonic growth modes.

Recently, Ma and colleagues (231) proposed that AlgC is a critical ‘check point’ enzyme in exopolysaccharide and B-band LPS production by *P. aeruginosa*. AlgC is a bifunctional enzyme with phosphomannomutase and phosphoglucomutase activities that convert mannose-6-phosphate and glucose-6-phosphate to mannose-1-phosphate and

glucose-1-phosphate respectively. Mannose-1-phosphate is the substrate of AlgA, PslB and WbpW, key phosphomannose isomerases that convert mannose-1-phosphate to GDP-mannose, a key precursor for alginate, Psl exopolysaccharide and B-band LPS production (49, 232). The authors suggest that the expression and activity of AlgC and competition for mannose-1-phosphate between different pathways can indirectly regulate alginate production at the posttranslational level (Fig. 2H).

Concluding remarks

Bacterial alginate biosynthesis is of great importance due to its role in the virulence of human and plant pathogenic *Pseudomonas* species, as well as its potential industrial applications. Although much has been learned over the past few decades, several key questions regarding the regulation of this polymer persist. What the specific environmental cues inducing alginate production in the various species are and the mechanisms behind how these signals are detected remain unclear. Furthermore, how the regulatory network is controlled with so much cross-talk between the regulators (e.g. AlgU, AmrZ and c-di-GMP are all heavily involved in other regulatory networks) remains an intriguing question.

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Chapter IV

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 is an indicator of poor prognosis for these patients. The proteins required for alginate polymerisation and secretion are encoded by genes organized in a single operon; however, the existence of internal promoters has been reported. It has been proposed that these proteins form a multiprotein complex which extends from the inner to outer membrane. Here, experimental evidence supporting such a multiprotein complex was obtained via mutual stability analysis, pulldown assays, and coimmunoprecipitation. The impact of the absence of single proteins or subunits on this multiprotein complex, i.e., on the stability of potentially interacting proteins, as well as on alginate production was investigated. Deletion of *algK* from an alginate-overproducing strain, PDO300, interfered with the polymerisation of alginate, suggesting that in the absence of AlgK, the polymerase and copolymerase subunits, Alg8 and Alg44, are destabilized. Based on mutual stability analysis, interactions between AlgE (outer membrane), AlgK (periplasm), AlgX (periplasm), Alg44 (inner membrane), Alg8 (inner membrane), and AlgG (periplasm) were proposed. Coimmunoprecipitation using a FLAG-tagged variant of AlgE further demonstrated its interaction with AlgK. Pulldown assays using histidine-tagged AlgK showed that AlgK interacts with AlgX, which in turn was also copurified with histidine-tagged Alg44. 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 (233, 234).

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 (9, 10). 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 (43, 47, 50). GDP-mannuronic acid is polymerised into a polymannuronate chain by the inner membrane glycosyltransferase, Alg8, with some involvement by Alg44 (53, 235, 236). Alg8 has multiple transmembrane domains and a large cytoplasmic glycosyltransferase domain (235) while Alg44 has a single transmembrane domain separating a cytoplasmic c-di-GMP binding PilZ domain and a large C-terminal periplasmic domain (236). 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 (16, 55, 57, 237, 238). 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 (23, 70). A component of the proposed periplasmic scaffold, AlgG, is an epimerase which converts D-mannuronic acid into α -L guluronic acids at polymer level (16, 239). AlgL, a bi-functional periplasmic protein, serves as an alginate degrading enzyme while also contributing to the integrity of the periplasmic scaffold (25, 237). 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 (19). 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 (79, 240). The exact functions of AlgK and AlgX are not clear; however, they are essential for alginate secretion (55, 238, 241).

The nascent alginate is secreted out of the cell through AlgE, which forms an electropositive β -barrel pore in the outer membrane (27, 57, 77).

Recent studies have suggested that proteins involved in the biosynthesis of alginate form a multiprotein complex which spans the entire bacterial envelope fraction (54). 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 (PDO300 Δ *algX*, PDO300 Δ *algK*, PDO300 Δ *alg44*, PDO300 Δ *algE*, PDO300 Δ *alg8* and PAO1 Δ *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

PDO300 Δ *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:*algKNC*. A 1,100 bp fragment, FRT-*aacCI*-FRT, encoding a gentamycin resistance gene (*aacCI*) flanked by FLP-recombinase recognition sites (FRT), was obtained by digesting the plasmid pPS856 with *BamHI* (242). The resulting fragment FRT-*aacCI*-FRT was inserted into pGEM-TEasy:*algKNC*, yielding the construct pGEM-TEasy:*algKNC* Ω Gm. The plasmid pGEM-TEasy:*algKNC* Ω Gm was used as a template for amplifying the *algKNC* Ω Gm 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* Ω Gm. This plasmid was transferred into *P. aeruginosa* PDO300 via conjugation utilising the *E. coli* S17-1 (243, 244). Transconjugants were

selected for on Mineral Salt Medium (MSM) supplemented with 100 µg/mL of gentamycin and 5% (wt/vol) sucrose (245). The double crossover event, generating the strain PDO300Δ*algK*ΩGm, was confirmed by gentamycin resistance (300 µg/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 (242) into PDO300Δ*algK*NCΩGm to remove the gentamycin resistance cassette (FRT-*aacCI*-FRT). Successful transconjugants were selected on Pseudomonas Isolation Agar (PIA) containing carbenicillin (300 µg/ml) and subsequently transferred to PIA plates containing 5% sucrose to remove the pFLP2 plasmid. Successful loss of the FRT-*aacCI*-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-6his* were generated as follows. The *algK* gene and a C-terminal hexahistidine 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 *Bam*HI and *Hind*III, releasing the fragments *algK*(HiSDNd-Ba) and *algK*(HiSNd-6xHBa), which were independently ligated into the corresponding sites of pBBR1MCS-5 (246) yielding the final constructs pBBR1MCS-5::*algK* and pBBR1MCS-5:*algK-6his* 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* (57) 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:*PmucD* the promoter region -901 bp relative to *algU* open reading frame was amplified using primers *palgUHindIII*F and *palgUXba*IR (247) and

hydrolysed with *HindIII* and *XbaI*, the *mucD* region was hydrolysed from pBBR1MCS-5:*mucD* (79) using *XbaI* and *SacI* and purified and ligated together with *algU* promoter in mini-CTX-*lacZ* (248) hydrolysed with *HindIII* and *SacI* generating miniCTX:*PmucD*. To generate plasmids miniCTX:*Palg44*, miniCTX:*Palg8*, miniCTX:*PalgK*, miniCTX:*PalgX*, and miniCTX:*PalgE* the *algD* promoter fragment was hydrolysed from pGEM-TEasy:*PalgD* with *PstI* and *HindIII* 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:*Palg44*, miniCTX:*Palg8*, miniCTX:*PalgX*, miniCTX:*PalgE*, miniCTX:*PmucD* and miniCTX:*PalgK*) were electroporated into PDO300 Δ *alg44*, PDO300 Δ *alg8*, PDO300 Δ *algX*, PDO300 Δ *algE*, PAO1 Δ *mucD* described in previous studies (53, 54, 57, 79, 249) as well as into PDO300 Δ *algK*, respectively. Transformants were selected for on PIA supplemented with 150 μ g/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 scraping 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 \times 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 \times 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 NaH₂PO₄·xH₂O, 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 μl of 1X SDS-PAGE loading buffer (62.5mM Tris-HCl (pH 6.8 at 25°C), 2% wt/vol SDS, 10% glycerol, 0.01% wt/vol 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 DNaseI 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-lauroyl 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% wt/vol) in Tris-buffered-saline containing Tween-20 (0.05% vol/vol) (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% wt/vol) 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 quantified as described previously (57). 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 (15). 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 μ l 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 (15).

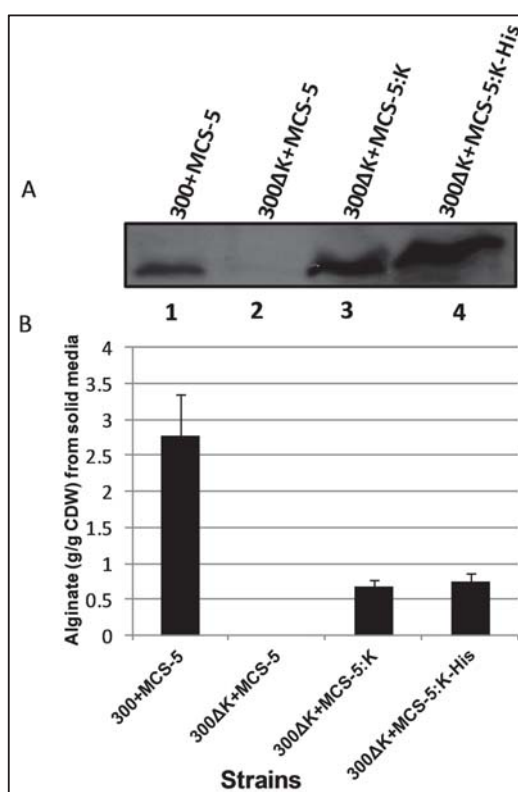
Results

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 PDO300 Δ *algK* showed that AlgK was absent (Fig. 1A). Similar to what has been described previously (55), 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 PDO300 Δ *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, PDO300 Δ *algK*+pBBR1MCS-5:*algK* and PDO300 Δ *algK*+pBBR1MCS-5:*algK*-6*his* (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

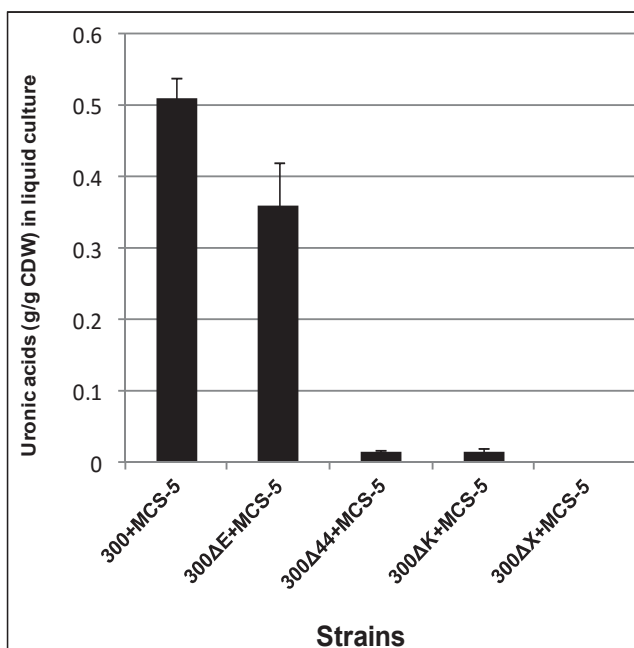


conducted in triplicates and error bar represents the standard deviation from the mean value. ENV: Envelope; IB: Immunoblotting; CDW: Cellular Dry Weight; SD: Standard deviation.

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 (16, 57, 238). 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 *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*, PDO300 Δ *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 PDO300 Δ *algK*(pBBR1MCS-5:*algK*) was 4-fold less than PDO300(pBBR1MCS-5) (Fig. 1B) and the alginate produced by PDO300 Δ *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 (54). 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 PDO300 Δ *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 PDO300 Δ *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.

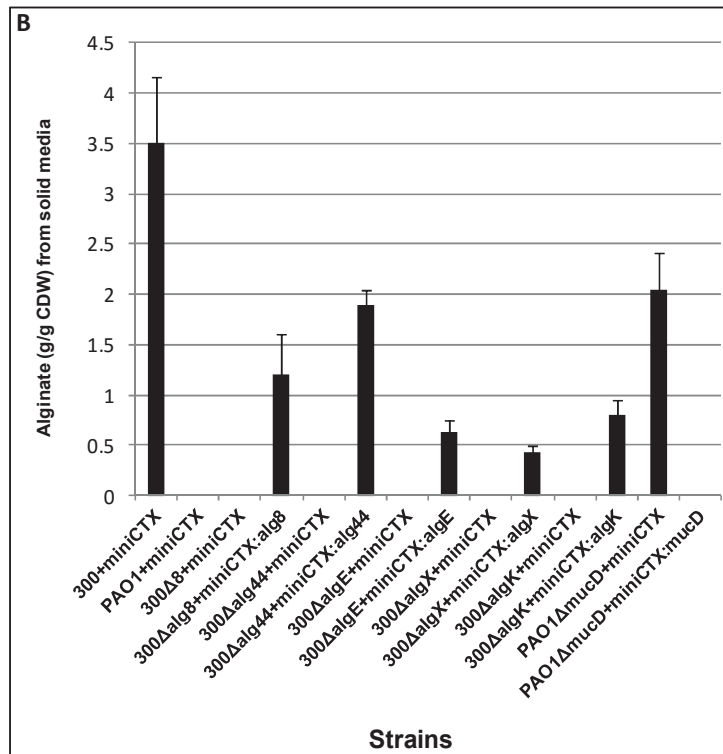
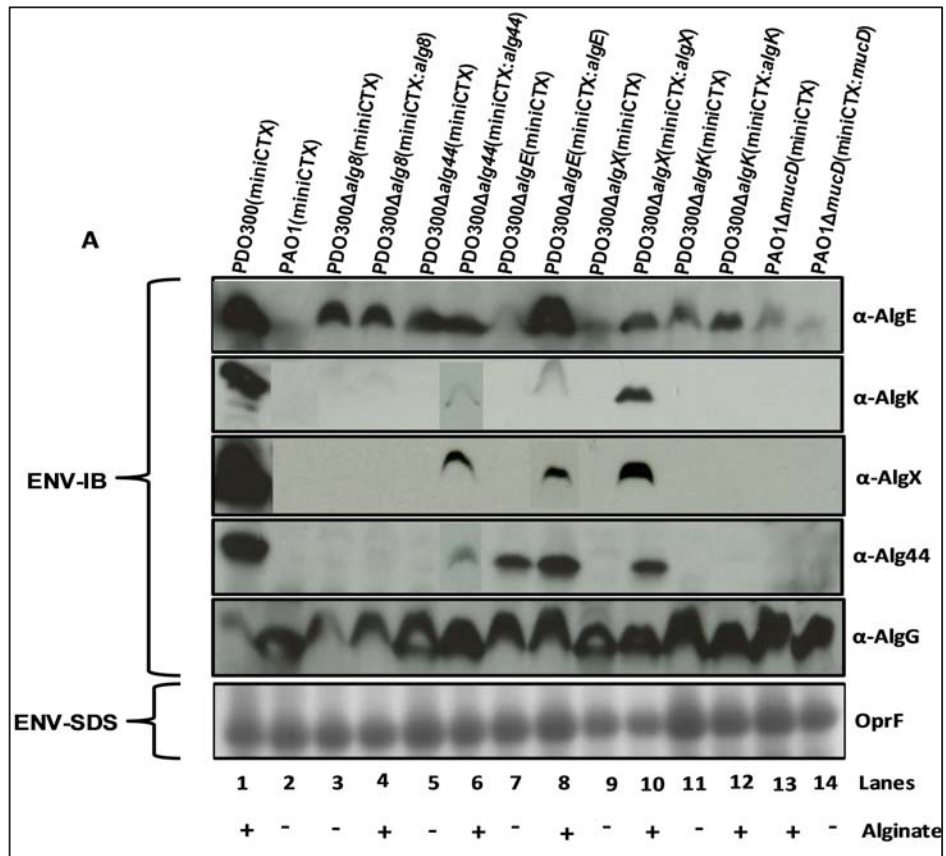


Fig. 3. Effect of the absence or presence of proposed subunits of the alginate biosynthesis machinery on the stability other subunits in the multiprotein complex and alginate production. (A) Envelope fractions from PDO300, PAO1, knock-out mutants of *alg8*, *alg44*, *algE*, *algX*, *algK* and *mucD* 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 (19, 79).

Interestingly, deletion of *algX* (PDO300 Δ *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 (79). 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 PDO300 Δ *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 PAO1 Δ *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 PDO300 Δ *alg8* with *alg8* or PDO300 Δ *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 (250) which suggested that on solid media the transcription from *algD* operon increases and/or the alginate biosynthesis multiprotein complex is more active/abundant.

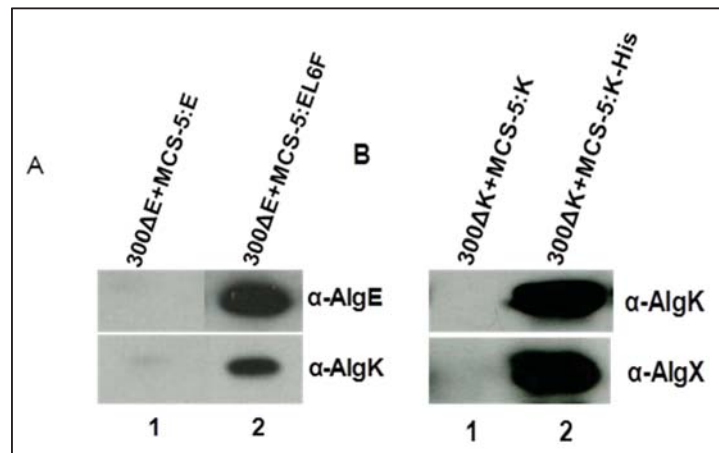
AlgK interacts with AlgE and AlgX *in vivo*

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 (19). 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 (251, 252). Despite these precedences no direct evidence had been provided for this long proposed interaction between AlgE and AlgK (19, 27). To investigate this further, a co-IP assay was performed by expressing in PDO300 Δ *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 PDO300 Δ *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 from 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* PDO300 Δ *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* PDO300 Δ *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 PDO300 Δ *alg44* (53) 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 Δ *alg44*(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* (PDO300 Δ *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.

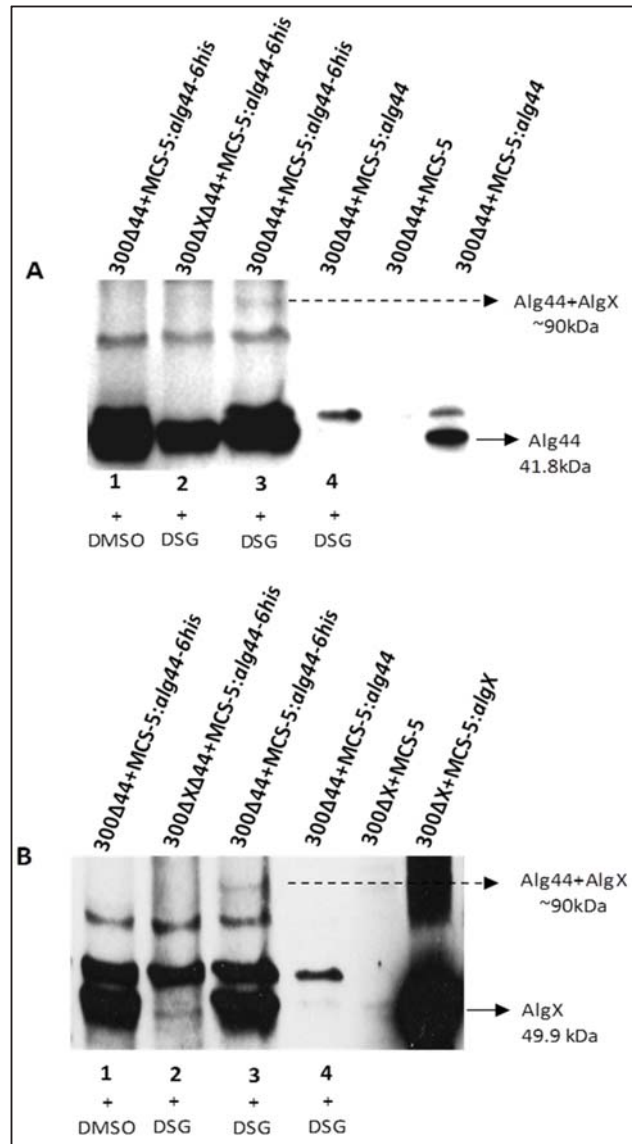


Fig. 5. *In vivo* cross-linking shows Alg44 interacts with AlgX. Cells were grown overnight in planktonic mode and incubated with cross-linker DSP (Lane 2, 3, 4 AB) or just DMSO (Lane 1 AB). Envelope fractions were isolated, detergent solubilised and subjected to affinity chromatography as mentioned 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 (19). This protein has multiple copies of the tetratricopeptide-repeat motif commonly found in proteins involved in the assembly of multiprotein complexes (19). 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 PDO300 Δ *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 (55). Interestingly, PDO300 Δ *algK* produced almost no free uronic acid oligomers while FRD1100 produced significantly more (Fig. 2) (55). 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 suggest 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, PDO300 Δ *algK* has no extra promoter or marker present. The yield of free uronic acids produced by PDO300 Δ *algK* was comparable to that of PDO300 Δ *algX*, and PDO300 Δ *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 (PDO300 Δ *algK*, PDO300 Δ *alg44*, and PDO300 Δ *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 PDO300 Δ *algE* (Fig. 3A lane 7) and that AlgK was required for the proper localisation of AlgE to the outer membrane (19) 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 (253). 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 (79) (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 (53, 235, 236). 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 (254). 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 PDO300 Δ *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 (53, 236). 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 (251, 255). 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 (PAO1 Δ *mucD*) leads to increased transcription of *algD* operon and overproduction of alginate (155, 256). 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 their own internal promoters (other than the promoter upstream of *algD*) (257). 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 (41). 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 (179). 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 (258). The role of AlgE in attachment to polystyrene microtitre plates was evaluated by using PAO1 Δ 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 (23). 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

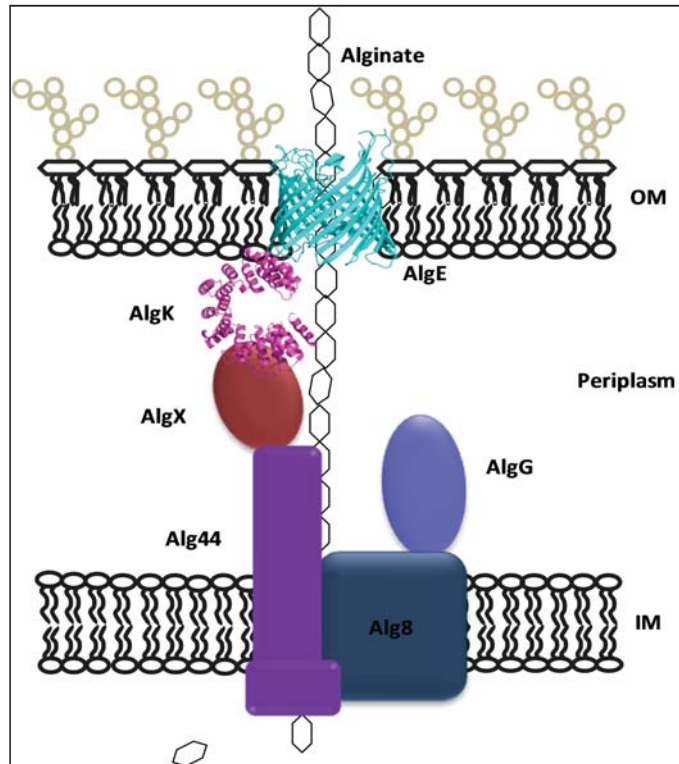


Fig. 6. Proposed model of the alginate polymerisation/secretion multiprotein complex. Based on results from mutual stability and pull-down assays, a model of the assembly for alginate polymerisation and secretion machinery was proposed. This model suggest that OM located AlgE interacts with periplasmic 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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We are thankful to Lynne Howell from Toronto University and Dennis E. Ohman from Virginia Commonwealth University for providing antibodies.

Supplementary Material

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

Strains or plasmids	Characteristics	source
Strains		
<i>P. aeruginosa</i> PAO1	Prototrophic wild type strain; Alg ⁻	(259)
<i>P. aeruginosa</i> PDO300	<i>mucA22</i> isogenic mutant derived from PAO1	(259)
PDO300Δ <i>algK</i>	Isogenic <i>algE</i> deletion mutant derived from PDO300	This study
PDO300Δ <i>alg8</i>	Isogenic <i>algE</i> deletion mutant derived from PDO300	(54)
PDO300Δ <i>alg44</i>	Isogenic <i>algE</i> deletion mutant derived from PDO300	(53)
PDO300Δ <i>algX</i>	Isogenic <i>algE</i> deletion mutant derived from PDO300	(249)
PDO300Δ <i>algE</i>	Isogenic <i>algE</i> deletion mutant derived from PDO300	(57)
PAO1Δ <i>mucD</i>	Isogenic <i>algE</i> deletion mutant derived from PDO300	(79)
<i>E. coli</i>		
TOP10	<i>E. coli</i> cloning strain	Invitrogen
S17-1	<i>thi-1 proA hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1</i> ; <i>tra</i> gene of plasmid RP4 integrated in chromosome	(244)
Plasmids		
pEX100T	Ap ^r Cb ^r , gene replacement vector containing <i>sacB</i> gene for counterselection	
pEX100T:Δ <i>algE</i> ΩGm	Ap ^r Cb ^r Gm ^r ; vector pEX100T with SmaI-inserted <i>algE</i> deletion construct	(57)
pEX100T:Δ <i>algK</i> ΩGm	Ap ^r Cb ^r Gm ^r ; vector pEX100T with <i>EcoRV</i> -inserted <i>algK</i> deletion construct	This study
pEX100T:Δ <i>algX</i> ΩGm	Ap ^r Cb ^r Gm ^r ; vector pEX100T with <i>EcoRV</i> -inserted <i>algX</i> deletion construct	(249)

pPS865	Ap ^r Gm ^r ; source of 1,100-bp <i>Bam</i> HI fragment comprising <i>aacC1</i> gene flanked by Flp recombinase target site signal sequences	(242)
pPFLP2	Ap ^r Cb ^r ; broad-host-range vector encoding Flp recombinase	(242)
pGEM [®] -T Easy	Ap ^r , P _{lac}	Invitrogen
pBBR1MCS-5	Gm ^r ; broad-host-range vector; P _{lac}	
pBBR1MCS-5: <i>alg44</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>alg44</i> inserted	(53)
pBBR1MCS-5: <i>algE</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>algE</i> inserted	(57)
pBBR1MCS-5: <i>alg44algX</i>	<i>Hind</i> III and <i>Cla</i> I fragment comprising <i>alg44</i> was ligated into pBBR1MCS-5: <i>algX</i> hydrolysed with same enzymes	
pBBR1MCS-5: <i>algK</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>algK</i> inserted	This study
pBBRMCS-5: <i>algEL6FLAG</i>	<i>algE</i> fragment with a 24 bp insertion (encoding the FLAG epitope) inserted after the 513 th bp of the ORF	(57)
pGEM-TEasy: <i>PalgD</i>	-879 bp region, flanked by <i>Pst</i> I and <i>Bam</i> HI, relative to the <i>algD</i> open reading from was amplified and ligated	This study
pGEM-TEasy: <i>PalgU</i>	-901 bp region, flanked by <i>Xba</i> I and <i>Hind</i> III, relative to <i>algU</i> open reading frame was amplified and ligated	This study
mini-CTX- <i>lacZ</i>	Integration proficient vector	(260)
miniCTX: <i>PalgK</i>	<i>algK</i> fragment with <i>algD</i> promoter cloned into chromosomal integration vector	This study
miniCTX: <i>Palg8</i>	<i>alg8</i> fragment with <i>algD</i> promoter cloned into chromosomal integration vector	This study
miniCTX: <i>Palg44</i>	<i>alg44</i> fragment with <i>algD</i> promoter cloned into chromosomal integration vector	This study

miniCTX: <i>PalgX</i>	<i>algX</i> fragment with <i>algD</i> promoter cloned into chromosomal integration vector	This study
miniCTX: <i>PalgE</i>	<i>algE</i> fragment with <i>algD</i> promoter cloned into chromosomal integration vector	This study
miniCTX: <i>PmucD</i>	<i>mucD</i> fragment with <i>algU</i> promoter cloned into chromosomal integration vector	This study

Table S2. Oligonucleotides used in this study.

Primers	Sequence
algKNF(EcoRV)	GATATCATGAAGATGCCATCCTCCCTCC
algKNR(BamHI)	GGATCCCGATTTCCGGCCAGGACTGC
algKCF(BamHI)	GGATCCTACTACCTGGGGCAGATCTA
algKCR(EcoRV)	GATATCCTCATAGGCTTTCTGGCTCTTC
algK(upXout)	ACCCTGCTGAACAAGGCCGTGAC
algK(downXout)	GCGGGTTGACGGAACGGGAGCTG
algKN(HiSDNd)	ACCAAAGCTTAGGAGAGAAAGCATATGAAGATGCC CATCCTCCCTCCCCTGC
algKC(BamHI)	GACGGATCCTCATAGGCTTTCTGGCTCTTCTTCG
algKC(6xhBamHI)	GTCTGGGATCCGACTTAATGATGGTGATGGTGGTGT AGGCTTTCTGGCTCTTCTTC GTTGATCGGCGAG
Pser Up	CGAGTGGTTTAAGGCAACGGTCTTGA
Pser Down	AGTTCGGCCTGGTGAACAACCTCG
palgUXbaIR	TCTAGAGAAAGCTCCTCTTCGAACCTGGAGG
palgUHindIIIF	AAGCTTCGACCGCCACCCGCTGCGAC

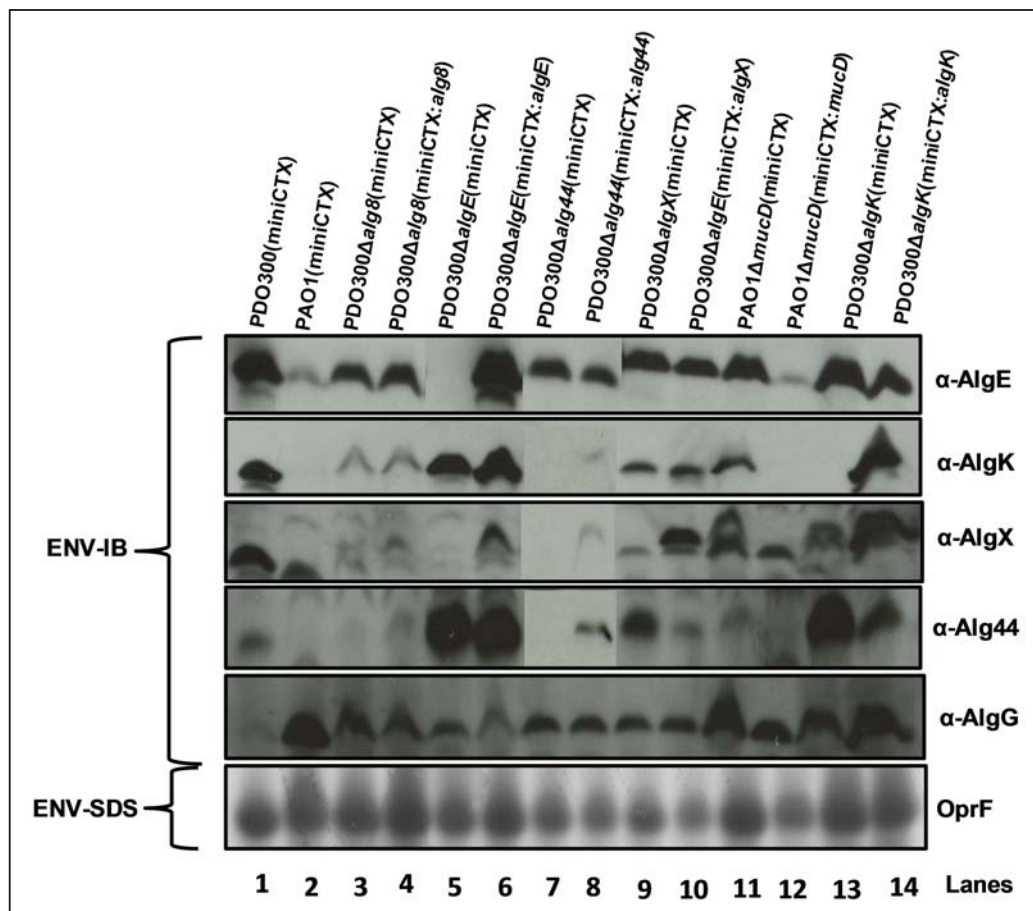


Fig. S1. Mutual stability analysis. Envelope fractions from PDO300, PAO1, knock-out mutants of *alg8*, *alg44*, *algE*, *algX*, *algK* and *mucD* and their *in cis* complemented strains, were isolated after 48 h growth on solid media and subjected to immunoblot analysis. The envelope fractions were probed 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 V

Alginate polymerisation and modification are linked in *Pseudomonas aeruginosa*

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Abstract

The molecular mechanisms of alginate polymerisation/modification/secretion by a proposed envelope spanning multiprotein complex are unknown. Here, bacterial two-hybrid assays and pull-down experiments showed that the catalytic subunit Alg8 directly interacts with the proposed co-polymerase Alg44 while embedded in the cytoplasmic membrane. Alg44 additionally interacts with the lipoprotein AlgK bridging the periplasmic space. Site-specific mutagenesis of Alg44 showed that protein-protein interactions and stability were independent of conserved amino acid residues R17 and R21, which are involved in c-di-GMP binding, the N-terminal PilZ domain and the C-terminal 26 amino acids. Site-specific mutagenesis was employed to investigate the c-di-GMP mediated activation of alginate polymerisation by the PilZ_{Alg44} domain and Alg8. Activation was found to be different to the proposed activation mechanism for cellulose synthesis. The interactive role of Alg8, Alg44, AlgG (epimerase) and AlgX (acetyltransferase) on alginate polymerisation and modification was studied by using site-specific deletion mutants, inactive variants and overproduction of subunits. The composition, molecular weight and materials properties of resulting novel alginates were analysed. The molecular weight was reduced by epimerisation while it was increased by acetylation. Interestingly, when overproduced, Alg44, AlgG and the non-epimerizing variant AlgG(D324A) increased the degree of acetylation while epimerisation was enhanced by AlgX and its non-acetylating variant AlgX(S269A). Biofilm architecture analysis showed that acetyl groups promoted cell aggregation while non-acetylated polymannuronate alginate promoted stigmergy. Overall, this study sheds new light on the arrangement of the multiprotein complex involved in alginate production. Furthermore, the activation mechanism and the interplay between polymerisation and modification of alginate were elucidated.

Importance

This study provides new insights into the molecular mechanisms of the synthesis of the unique polysaccharide, alginate, which is not only an important virulence factor of the opportunistic human pathogen *Pseudomonas aeruginosa* but which has, due to its

material properties, many application in medicine and industry. Unraveling the assembly and composition of the alginate synthesizing and envelope spanning multiprotein complex will be of tremendous significance for the scientific community. We identified a protein-protein interaction network inside the multiprotein complex and studied its relevance with respect to alginate polymerisation/modification as well as the c-di-GMP mediated activation mechanism. A relationship between alginate polymerisation and modification was shown. Due to the role of alginate in pathogenesis as well as its unique material properties harnessed in numerous applications, results obtained in this study will aid the design and development of inhibitory drugs as well as the commercial bacterial production of tailor-made alginates.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen which can become life-threatening in immunocompromized patients. It is the leading cause of morbidity and mortality in cystic fibrosis patients. This is mainly due to its ability to colonize lungs by forming structured biofilms which consist of bacterial cells embedded in a complex matrix predominantly composed of alginate. Bacterial cells in biofilms are protected against the immune system and antibiotics (1, 2). Alginates are anionic exopolysaccharides composed of variable proportions of 1,4-linked β -D-mannuronic acid (M) and its C5 epimer α -L-guluronic acid (G). The alginate derived from *P. aeruginosa* is naturally acetylated and lacks consecutive G residues (GG-blocks) (7). Alginates exhibit unique gel-forming properties suitable for numerous medical and industrial applications (7, 8). The alginate structure strongly impacts its materials properties. Hence, development of bioengineering approaches to control the alginate structure will enable production of alginates with new material properties towards novel applications.

For many years *P. aeruginosa* has been the model organism to study various aspects of alginate biosynthesis such as e.g. polymerisation, epimerisation, acetylation, secretion and regulation. Thirteen proteins are directly involved in the biosynthesis of alginate and except for *algC* their encoding genes are clustered in the alginate biosynthesis operon (*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, *algA*) (9, 10). Except for soluble cytoplasmic proteins AlgA, AlgC and AlgD which are responsible for providing the activated nucleotide sugar precursor, GDP-mannuronic acid, proteins

encoded by the operon are proposed to constitute an envelope-spanning multiprotein complex. Two cytoplasmic membrane-anchored proteins, the glycosyltransferase, Alg8, and the proposed co-polymerase, Alg44, are necessary for alginate polymerisation (11-13). The MucR sensor protein, a diguanylate cyclase (DGC)/phosphodiesterase (PDE) embedded in the cytoplasmic membrane was proposed to provide c-di-GMP for binding to the cytoplasmic PilZ domain of Alg44 by which alginate polymerisation is activated (14). Translocation of nascent alginate across the periplasm is coupled with modification processes including O-acetylation and epimerisation. O-acetylation is independently catalyzed by AlgJ and AlgX (22) while the acetyl group donor is provided by AlgI and AlgF (23, 24). The AlgG epimerase converts M residues to G residues in the nascent alginate chain. AlgG, AlgX and AlgK were suggested to form a periplasmic scaffold for guiding alginate through the periplasm for secretion via the outer membrane protein AlgE (16-21). It was also suggested that if alginate is misguided into the periplasm then degradation would be mediated by the periplasmic AlgL lyase (25). Previous studies on protein-protein interactions and mutual stabilities of proposed subunits of the multiprotein biosynthesis machinery provided evidence of binary protein interactions including AlgE-AlgK, AlgX-AlgK, AlgX-MucD (a serine protease), Alg44-AlgX and Alg8-AlgG (79, 80). However, more experimental evidence is needed to map all protein-protein interactions within the multiprotein complex in particular towards unraveling the molecular mechanisms of alginate polymerisation, molecular weight control and the relationship of modification events to polymerisation.

In this study, protein-protein interactions within the multiprotein complex were investigated using the bacterial two-hybrid technique and pull-down assays. The proposed interacting protein surface of Alg44 was probed and the molecular mechanism of c-di-GMP mediated activation was studied (15, 261). The role of Alg8, Alg44, AlgG and AlgX with respect to polymerisation and modification was studied by analyzing the composition and material properties of alginates produced by various strains. We employed a constitutive alginate-producing strain *P. aeruginosa*, PDO300, to generate isogenic single- and double-gene knockouts of *alg8*, *alg44*, *algG* and *algX*. This allowed studying the role of the respective proteins in alginate polymerisation and/or modifications by introducing additional copy of subunits or their variants *in trans*. The impact of various alginate structures on motility, biofilm formation and architecture was investigated.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are summarized in Table S1. *P. aeruginosa* and *Escherichia coli* strains were grown at 37°C. For those assays designed for studying alginate production, bacterial phenotype and protein-protein interaction and stability, Difco™ Pseudomonas Isolation Agar (PIA) medium was used. To rule out the effect of growth condition and media on alginate composition and quantity, all experiments were conducted under the same conditions and using the same batch culture at the same time (see supplemental material).

Construction of isogenic single- and double-gene knockout mutant in *alg8*, *alg44*, *algG* and *algX* genes

The *P. aeruginosa* PDO300 was used to generate isogenic single- and double-gene knockout mutants in *alg8*, *alg44*, *algG* and *algX* genes through two events of homologous recombination using suicide plasmid pEX100T containing knockout genes which were disrupted by the *aacCI* gene (encoding gentamicin acetyltransferase) flanked by two *FRT* sites (see supplemental material).

***In trans*-complementation of single- and double-gene knockout mutants and chromosomal integrations**

Relevant genes encoding Alg8, Alg44, AlgG and AlgX (with and without 6his-tag) were individually or in combination transferred into generated mutants using pBBR1MCS-5 plasmid and also incorporated into the genome using mini-CTX-*lacZ* plasmid (see supplemental material). The pBBR1MCS-5 containing the various alginate genes were considered to study the impact on production of alginates and their characteristics in order to more sensitively detect changes in the alginates due to additional copy of the respective alginate protein under investigation.

Site-specific mutations and deletions of *alg44* and *alg8*

Site-specific mutations and deletions of *alg44* and *alg8* genes were performed using QuikChange[®] II Site-Directed Mutagenesis Kit (Stratagene) or by DNA synthesis (GenScript), resulting in genes encoding Alg8(E322A/H323/E326A), Alg44(R17A/R21A/R21D) and its N- and C-terminal truncation (see supplemental material).

***In vivo* detection of protein-protein interaction network**

In vivo detection of protein-protein interactions was performed by pull-down assays using native 6his-tagged proteins under native condition, *in vivo* chemical cross-linking and bacterial two-hybrid assay (see supplemental material).

Assessment of the stability of Alg44 variants in the presence and absence of MucR (DGC/PDE)/rocR (PDE)

Previously generated mutants including PDO300 Δ *mucR*, PDO300 Δ *mucR* (pBBR1MCS-5:*mucR*), PDO300 (pBBR1MCS-5:*rocR*), PDO300 Δ *mucR* (pBBR1MCS-5:*rocR*) were used to show positive or negative regulation of alginate production through c-di-GMP synthesis (by MucR) or degradation (by RocR) (15). Also to use presumably nonfunctional Alg44 in binding to c-di-GMP and/or in alginate polymerisation (12, 262), the mutants PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*(R21D)-6his), PDO300 Δ *alg44*(pBBR1MCS-5:*alg44*-6his(Δ 40-74aa_{pilZ})) and PDO300 Δ *alg44*(pBBR1MCS-5:*alg44*-6his(Δ 364-389aa)) were generated. Using these mutants we examined direct effect of c-di-GMP and defective variants of Alg44 on its localization and stability in planktonic and biofilm cells. Cells grown on solid media or in liquid cultures as described above were washed twice with saline. Enzymatic cell lysis was performed using abovementioned lysis buffer prepared in buffer A followed by sonication and isolation of cell envelope fraction by ultracentrifugation at 100,000 \times g for 1 h at 4 °C. Pellets were solubilized with buffer A for protein analysis.

Isolation of cytoplasmic membrane and general protein analysis

To confirm the localization of Alg8 and Alg44 in the cytoplasmic membrane of *E. coli* BTH101, the cytoplasmic membrane fraction was isolated as described previously with some modifications (263) (see supplemental material).

Protein samples were generally analysed utilizing SDS-PAGE (8% acrylamide gels) and Immunoblotting (see supplemental material).

Alginate purification and quantification

Two ml of bacterial overnight culture grown in LB medium supplemented with the appropriate antibiotic were sedimented and cells washed twice with saline solution. Cells were suspended in 1 ml of saline solution and 200 μ l of cell suspension was plated onto PIA medium (in triplicates) containing 300 μ g ml⁻¹ of gentamicin and then incubated at 37 °C for 72 h. Cells of each agar plate were scraped off and suspended in saline solution until the biomass were completely suspended. Then suspensions were pelleted and alginates in supernatants were precipitated with equal volume of ice-cold isopropanol. The alginate precipitants were freeze-dried and then re-dissolved in 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂ to a final concentration of 0.5% (wt/vol), followed by incubation with 15 μ g ml⁻¹ DNaseI and 15 μ g ml⁻¹ RNaseI at 37 °C for 6 h. Then, Pronase E was added to a final concentration of 20 μ g ml⁻¹ and incubated for further 18 h at 37 °C. Alginate solutions were dialyzed (12-14 kDa MWCO, ZelluTrans/Roth mini dialyzer, Carl Roth GmbH & Co) against 5 l of ultrapure H₂O for 48 h. Finally, alginates were precipitated with equal volume of ice-cold isopropanol and freeze-dried for uronic acid assay and biochemical analysis (see supplemental material).

The analysis of molecular weight, composition and viscoelastic properties of the alginates

Various alginates produced by different complemented mutants were subjected to molecular weight analysis using size exclusion chromatography-multiangle laser light scattering (SEC-MALLS) and compositional analysis utilizing ¹H-nuclear magnetic resonance (NMR) spectroscopy and Fourier transform infrared (FTIR) spectrometry (see Expanded View Materials and Methods). Microrheological analysis was used to measure viscoelastic property of the alginates in which the mean-square displacement (MSD) of probe particles embedded in the samples and in turn the viscoelastic moduli (G' (elastic) and G'' (viscose)) were measured (see supplemental material).

Continuous-culture flow cell biofilms, quantitative analysis and motility assays

Biofilm architecture analysis was performed for those mutants producing alginates with very distinct composition and properties from each other including

PDO300(pBBR1MCS-5), PDO300 Δ *alg8*(pBBR1MCS-5:*alg8*),
PDO300 Δ *alg44*(pBBR1MCS-5:*alg44*), PDO300 Δ *algG*(pBBR1MCS-5:*algG*),
PDO300 Δ *algG*(pBBR1MCS-5:*algG*(D324A)), PDO300 Δ *algX*(pBBR1MCS-5:*algX*),
PDO300 Δ *algX*(pBBR1MCS-5:*algX*(S269A)) and PDO300 Δ *algX* Δ *algG*(pBBR1MCS-
5:*algX*(S269A):*algG*(D324A)). Each mutant was grown in continuous-culture flow cells
(channel dimensions of 4 mm by 40 mm by 1.5 mm) at 37 °C (264). The flow cells
were then incubated at 37 °C for 24 h. Biofilms were stained utilizing the LIVE/DEAD
BacLight bacterial viability kit (Molecular Probes) and visualized using confocal laser
scanning microscopy (Leica SP5 DM6000B). For quantitative analysis of biofilms
IMARIS image analysis software (Bitplane) was employed. Biofilm architecture and
appearance, biovolume (μm^3), the ratio of biovolume per unit area ($\mu\text{m}^3 \mu\text{m}^{-2}$), dead-to-
live ratio, compactness and thickness of base layers were analysed (138, 265, 266).
Motilities including twitching, swarming and swimming were assessed by the method
explained by Pang *et al* (267) with modification. Full experimental details are provided
in supplemental materials.

Results

Protein-protein interaction of membrane-anchored Alg8 and Alg44 towards constitution of an active alginate polymerase subunit

The two cytoplasmic membrane-anchored proteins Alg8 and Alg44 were previously shown to be necessary for alginate polymerisation. Alg8 is a glycosyltransferase catalyzing alginate polymerisation, using the substrate GDP-mannuronic acid, while the c-di-GMP-binding PilZ domain containing Alg44, was proposed as co-polymerase (13, 268). However, the functional and structural interaction of Alg8 and Alg44 had not been elucidated. Therefore, the marker-free isogenic double-gene knockout mutant PDO300 Δ alg8 Δ alg44 was generated. This mutant lost the mucoid phenotype while introduction of plasmid pBBR1MCS-5:alg44:alg8 restored alginate production and the mucoid phenotype.

In order to investigate the proposed interaction of Alg8 and Alg44, functional His-tagged variants (Alg44-6his and Alg8-6his) were subjected to pull-down experiments under native conditions and to bacterial two-hybrid system assays. In pull-down experiments, wild-type Alg44 and Alg8 without His-tag served as negative controls. To address possible stoichiometric effects i.e. effects of increased copy numbers of individual subunits on the integrity of the multiprotein complex, single genes encoding Alg8-6his or Alg44-6his under the control of their native promoter were integrated into the genome. In contrast, *in trans* genes were present on plasmids in multiple copies under control of the strong constitutive *lac* promoter. Immunoblots showed that Alg44 with an apparent molecular weight of 41.8 kDa was co-purified with Alg8-6his produced either from *in trans* or *in cis* encoding genes and similarly Alg8 (~53 kDa) was co-purified with Alg44-6his while respective proteins were not detected for complemented mutants with native Alg44 and Alg8 as well as in double knockout mutants with single Alg8-6his or Alg44-6his (Fig. 1A, B).

In addition, the bacterial two-hybrid system showed that the chimeric enzyme adenylate cyclase was reconstituted when its two complementary fragments (T18/T25) were brought together by Alg8 and Alg44 interaction. β -galactosidase activity in those cells harboring two plasmids producing fusion proteins of Alg8 and Alg44

(pKNT25:*alg8*+pUT18:*alg44*) was on average 11-fold [672 U/mg of cellular dry weight] greater than the negative controls without the fusion protein partner (background control) or when compared with single fusion protein Alg8 or Alg44 [58-66 U/mg of cellular dry weight] as well as the vice-versa combination (pKNT25:*alg44* + pUT18:*alg8*) which showed a β -galactosidase activity of 160 U/mg of cellular dry weight (Fig. 1C) which was 2.7-fold greater than background control. Analysis of cytoplasmic membrane proteins by immunoblotting confirmed that both proteins were localized to the cytoplasmic membrane of *E. coli* (Fig. 1D). These results provided the first experimental evidence for the direct interaction between the membrane-anchored proposed alginate polymerase (glycosyltransferase) Alg8 and the co-polymerase Alg44.

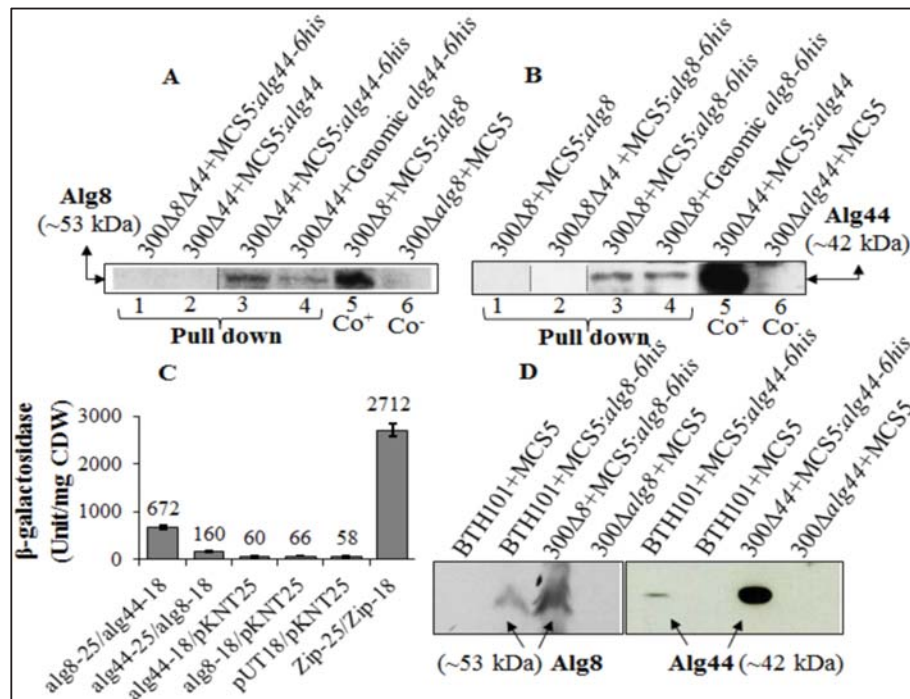


Fig. 1. Alg8/Alg44 protein-protein interaction. (A) and (B) His-tag based pull-down assays (lanes 1-4) and immunoblotting using anti-Alg8 antibodies (A) and anti-Alg44 antibodies (B) show protein-protein interaction between Alg8 and Alg44. Alg8 and Alg44 interaction was detected by respective co-purification of the non-His-tagged interacting protein partner encoded by genes either integrated into the genome or localized on a plasmid (lanes 3, 4). Lanes 1 and 2 belong to negative controls (proteins without His-tag or present individually in double-gene knockout mutants) (see Materials and Methods). (C) In bacterial two-hybrid analysis the appropriate pUT18 and pKNT25 derivatives were co-transferred into *E. coli* BTH101. The pUT18 and pKNT25 vectors were used as a negative control (background) in addition to transformants with one of the constructs and one empty vector, whilst the pUT18c-Zip and pKT25-Zip plasmid-pair was used as a positive control. Upon induction with 0.5 mM IPTG, fourfold β -

galactosidase activity higher than background was regarded as evidence for protein-protein interaction. (D) Immunoblots developed using anti-His-tag antibodies show production and subcellular localization of Alg8 and Alg44 to the cytoplasmic membrane of *E. coli* BTH101 (see Materials and Methods). 300: PDO300; 8, 44: Alg8, Alg44; MCS5: pBBR1MCS-5; Co+/-: positive/negative control; CDW: cellular dry weight; -18: pUT18; -25: pKNT25. An irrelevant lane was deleted between lanes 2 and 3 in blot A, as indicated by a thin line. Lane 2 in blot B was inserted as a representative control blot lane, as it was obscured by artificial stains in the original blot. Insertion of lane 2 is indicated by thin lines. Between dividing lines in blot B, a representative control blot lane was inserted from a separate blot, because in the original blot, it was obscured by artificial dots/development artifacts.

Cytoplasmic membrane-anchored Alg44 interacts with outer membrane-anchored AlgK while Alg44 is critical for structural integrity of the multiprotein alginate biosynthesis machinery

To assess whether Alg8 and Alg44 interact with other proposed subunits of the multiprotein complex, pull-down assays under native conditions were employed using Alg8-6his and Alg44-6his proteins followed by immunoblotting using anti-AlgX, -AlgK and -AlgE antibodies. Additionally, to rule out indirect interactions, appropriate double-gene knockout mutants harboring individual genes *in trans* (PDO300 Δ *alg8* Δ *alg44* (pBBR1MCS5:*alg8*-6his), PDO300 Δ *alg8* Δ *alg44* (pBBR1MCS5:*alg44*-6his), PDO300 Δ *alg44* Δ *algX* (pBBR1MCS5:*alg44*-6his)) were included. To address stoichiometric effects, complemented PDO300 Δ *alg8* and PDO300 Δ *alg44*, respectively, were used to generate single gene copy complementation strains by integrating *alg8*-6his or *alg44*-6his into the bacterial genome, respectively. The mutants producing native Alg8 and Alg44 proteins were used as negative controls. Resultant immunoblots (Fig. 2A, B) showed AlgK and AlgX but not AlgE (data not shown) were independently pulled down with Alg44-6his, both when *alg44*-6his was provided *in trans* or *in cis*. Hence, experimental evidence is provided as previously proposed (80) that a protein-protein interaction network spanning the periplasm and constituted by Alg8-Alg44-AlgK-AlgE interactions exists.

Cross-linking experiments using DSG cross-linking reagent with spacer arm length of 7.7 Å followed by Alg44-6his pull-down under denaturing condition showed a protein with an apparent molecular weight of ~84 kDa which was only detected the anti-Alg44 antibody. In addition the previously shown Alg44-AlgX (80) interaction was confirmed by detecting a cross-linked protein with an apparent molecular weight of ~90 kDa

binding both anti-Alg44 and anti-AlgX antibodies. The ~84 kDa protein was only detected in pull-down elution fractions obtained from genomic expression of *alg44-6his*, but not from the plasmid-borne gene, while Alg44-AlgX interactions were found to be independent of the stoichiometry of the individual proteins (Fig. 2C, D). These proteins were not detected in elution fractions when the native protein Alg44 was present and in the negative control treated with DMSO.

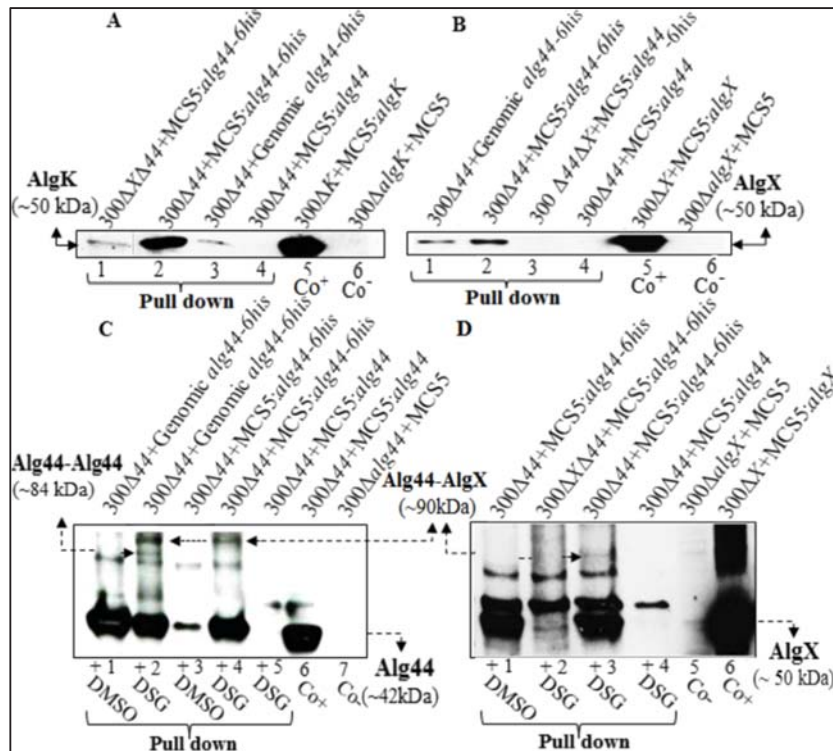


Fig. 2. Protein-protein interaction analysis indicates interaction of Alg44-AlgK, Alg44-AlgX and probable Alg44-Alg44 (dimer). (A) and (B) His-tag based pull-down assays and immunoblotting using anti-AlgK (A) and anti-AlgX (B) antibodies show interactions of Alg44-AlgK and Alg44-AlgX (A and B, lanes 1-4). These interactions were detected in both cases of *in trans* and *in cis* complementation of mutants with *alg44-6his* (lanes 2, 3 of blot A; lanes 1, 2 of blot B). AlgK was pulled down using His-tagged Alg44 in the absence of AlgX (lane 1, blot A). The elution fraction derived from complemented mutants producing non-His-tagged protein lacked AlgK and AlgX (lane 4 in A, B). (C) and (D) Immunoblots suggest *in vivo* Alg44 dimerisation and Alg44-AlgX interaction using stabilization of interaction by chemical cross-linking combined with His-tagged Alg44 mediated pull-down assays under denaturing conditions (see Materials and Methods). A presumable Alg44 dimer band (~84 kDa) reacted only with anti-Alg44 antibody (C, lane 2) while Alg44-AlgX band (~90 kDa) reacted with Alg44 and AlgX antibodies in both blots (blot C, lane 2, 4 and blot D, lane 3). These bands were not detected in the elution fraction derived from mutants with native Alg44 with DSG and Alg44-6his treated with DMSO as negative controls (blot C, lanes 1, 3, 5; blot

D, lanes 1, 4). 300: PDO300; MCS5: BBR1MCS-5; Co+/-: positive/negative control. An irrelevant lane was deleted between lanes 1 and 2 in blot A, as indicated by a thin line.

Alg44 variants with truncated PilZ domain and C-terminus were stable and maintained integrity of protein-protein interactions within the alginate biosynthesis multiprotein complex

Previously it was demonstrated that site-directed mutagenesis of the putative c-di-GMP-binding motifs (R17XXXR21) of the PilZ domain and a C-terminal truncation of Alg44 completely abolished alginate production (262). In comparison, it was shown that c-di-GMP binds directly to both PgaC and PgaD, the two cytoplasmic membrane components of the *E. coli* poly- β -1,6-N-acetylglucosamine synthesis machinery, which stimulated their glycosyltransferase activity by stabilizing their interaction (227). Here it was investigated whether c-di-GMP binding to the PilZ domain of Alg44 and the C-terminal part itself impact on protein-protein interactions and ultimately alginate polymerisation.

His pull-down experiments under native condition as described above were applied using His-tagged Alg44 variants (Alg44 (R21D), Alg44 (Δ 40-74aa_{PilZ}) and C-terminally truncated Alg44 (Δ 364-389aa)) (Fig. 3A). As shown in Fig. 3B, Alg44-6his variants were all found in the envelope fraction which suggested their localization and stability was not affected. Interestingly, the abovementioned protein interaction network was confirmed which signifies neither protein stability nor the interaction of Alg44 with AlgK and AlgX were disrupted by its defective PilZ domain and the C-terminal truncation respectively. However, the C-terminal truncation of Alg44 impacted on the stability of Alg8 (Fig 3C, lane 1), while the other variants of Alg44 did not (Fig. 3C).

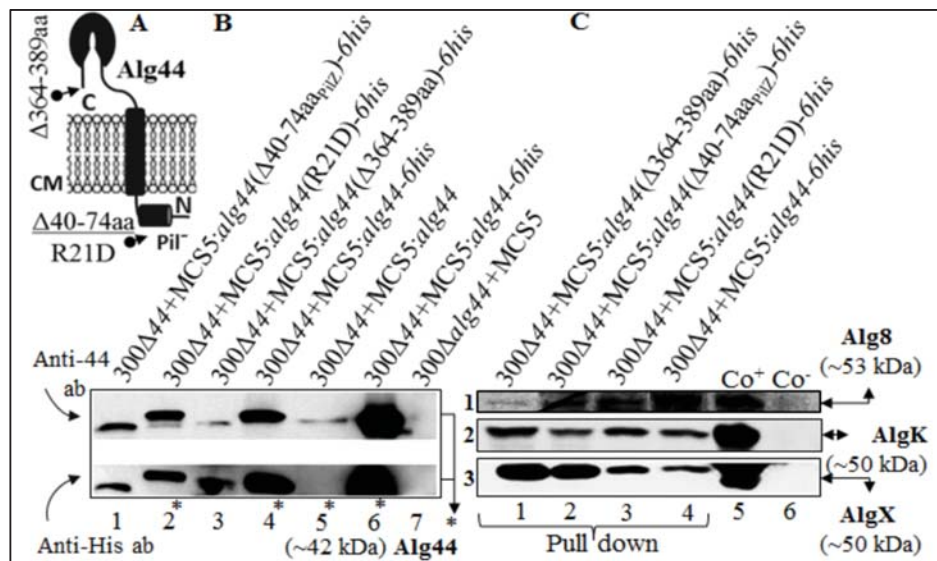


Fig. 3. Localization, stability and protein-protein interaction of Alg44 variants. (A) Schematic view of deleted or point-mutated region of Alg44. (B) Immunoblots of the envelope fraction developed using anti-Alg44 (upper) and anti-His-tag (lower) antibodies showed that with the various Alg44 variants localized to the envelope fraction (immunoblot B, lanes 1-3). Lanes 4-7 represent negative and positives controls. (C, blots 1-3, lanes 1-4) Immunoblots suggested protein-protein interaction of Alg44 variants with Alg8, AlgK and AlgX by using pull-down experiments using His-tagged variants of Alg44. C-terminal deletion of Alg44 had a destabilizing effect on Alg8. Immunoblots were developed using anti-Alg8 (1), anti-AlgK (2) and anti-AlgX (3) antibodies, respectively. Asterisks indicate full length Alg44. 300: PDO300; MCS5: pBBR1MCS5; Ab: antibody; Co^{+/-}: positive/negative control.

C-di-GMP levels and growth mode impact on Alg44 stability

Previous studies showed that introducing high copy numbers of *mucR* in PDO300Δ*mucR* resulted in greater production of alginate than found in the wild-type while increased copy numbers of *rocR* encoding a c-di-GMP degrading phosphodiesterase (PDE) led to strongly reduced alginate production presumably due to reduction in c-di-GMP levels. Therefore it was suggested that MucR plays a specific role in the regulation of alginate biosynthesis by co-localizing with Alg44 and providing a localized c-di-GMP pool (14). Here it was tested if Alg44 copy numbers in the envelope fraction might be affected by presence or absence of MucR and RocR,

respectively, i.e. by different c-di-GMP levels and within different physiological conditions such as planktonic and biofilm growth modes. Immunoblotting analysis of envelope fractions of the various mutants showed the amount of Alg44 was not significantly affected by the absence of MucR in biofilm mode while it was reduced in envelope fraction of biofilm cells with high copy number of *rocR* in the absence of MucR (see Fig. S1 in the supplemental material), indicating that the low amount of Alg44 corresponded with low c-di-GMP levels. However the amount of Alg44 in the same mutants growing in planktonic mode did not significantly differ.

Is alginate polymerisation controlled by an autoinhibition mechanism as shown for the bacterial cellulose synthase?

Alg8 and BcsA of bacterial cellulose synthase both belong to the glycosyltransferase family-2 (GT-2) and they share the same conserved signature motifs and residues experimentally known as critical for production of alginate and cellulose, respectively (64, 269). Recently, the structure of the bacterial cellulose synthase BcsA-BcsB complex was resolved. It was shown that the PilZ domain in this complex was in proximity to the catalytic site of BcsA (270). The first arginine of the PilZ domain's R580XXXR584 motif formed a salt bridge with E371 preceding the RW motif (a signature of glycosyltransferase-2 family), consequently tethering the gating loop in the resting status and blocking the catalytic site. This steric hindrance was called autoinhibiting mechanism which was proposed to be eliminated upon c-di-GMP binding to R580 opening up the gate for precursors to enter into the catalytic site.

Accordingly, informed by BcsA-BcsB structure and implementing bioinformatics analysis using the Phyre2 Protein Fold Recognition Server, an *in silico* structural model of Alg8 fused with the C-terminal PilZ_{Alg44} domain was developed (271). A structural model homologous to BcsA (confidence: 100%, coverage: 93%) showed the PilZ domain in proximity to the catalytic site of Alg8 and close to the residues E322, H323 and E326 located on BcsA-homologous loop preceding motif RW(339-340), a site potentially involved in salt-bridge formation (Fig. 4A-C). The impact of alanine substitutions of these residues, individually and in combination with R17 and/or R21 of Alg44 RXXXXR (17-21) motif at different c-di-GMP levels (i.e. presence or absence of overproduced c-di-GMP degrading RocR), on *in vivo* activity of respective Alg8 and Alg44 variants was assessed. Overproduction of RocR was confirmed to significantly reduce alginate production in the wild-type strain and complemented mutants.

Substitution of R residues in PilZ_{Alg44} domain's RXXXR motif (17-21) and E322 residue of Alg8 with alanine, respectively, completely abolished alginate production. The mutagenesis of H323 (i.e. PDO300Δ*alg8* (pBBR1MCS-5:*alg8*(H323A))) lowered alginate production by 6.9-fold when compared to PDO300Δ*alg8* (pBBR1MCS-5:*alg8*). Interestingly, RocR mediated reduced intracellular c-di-GMP levels restored alginate production to PDO300Δ*alg8* (pBBR1MCS-5:*alg8:rocR*) levels (Fig. 4D, labeled with asterisk). Replacement of E326 by alanine in Alg8 increased alginate production by 1.3-fold when compared with PDO300Δ*alg8* (pBBR1MCS-5:*alg8*). RocR production in this mutant background mediated decreased alginate production by about 2-fold when compared to wild-type Alg8 (i.e. PDO300Δ*alg8* (pBBR1MCS-5:*alg8:rocR*)) (Fig. 4D). In summary, conserved R residues of Alg44 proposed to be involved in autoinhibition via salt bridge formation inactivated alginate polymerisation. However, the replacement of H323 or E326 of Alg8 still mediated alginate production while reduced levels of c-di-GMP did cause less or no reduction alginate production when compared to the reference strain (PDO300Δ*alg8* (pBBR1MCS-5:*alg8*)).

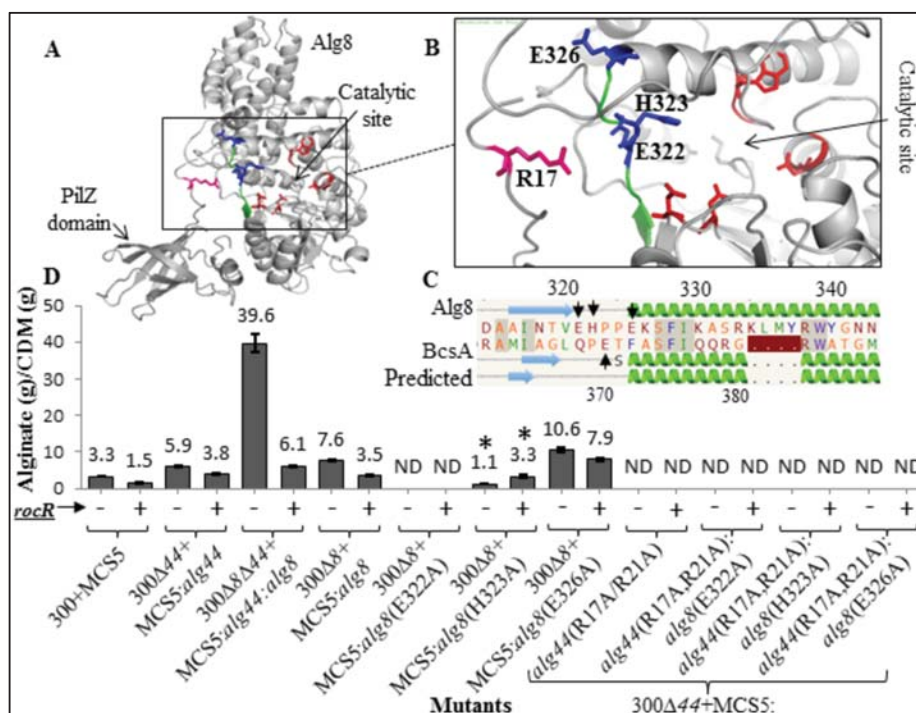


Fig. 4. Bacterial cellulose synthase-associated autoinhibiting mechanism does not play a role in alginate polymerisation. (A) *In silico* fusion of Alg8-PilZ_{Alg44} was modelled using Phyre2 server. (B) Highly conserved amino acids (blue sticks, E322, H323 and E326) are labelled with arrows in pairwise alignment of Alg8 with BcsA (C) were identified in a similar position to that of BcsA and which could form a salt bridge with R residues of PilZ_{Alg44} domain's R17XXXR21 motif and were chosen to be

replaced by alanine using site-specific mutagenesis. (D) Alginate quantification of PDO300 Δ *alg8*, PDO300 Δ *alg44* and PDO300 Δ *alg8* Δ *alg44* transformants with plasmids harboring respective site-specific mutants of *alg8* and *alg44* with (+) and without (-) *rocR* gene. Alg8's mutated residue which positively responded to c-di-GMP level alteration by RocR is labeled with asterisk. 300:PDO300; ND: Not detectable; MCS5: pBBR1MCS-5.

Interplay of alginate polymerizing (Alg8-Alg44) and modifying units (AlgG-AlgX) on alginate composition and molecular weight

In order to investigate the relationship between alginate polymerisation and modification, single- and double-gene knockout mutants of *P. aeruginosa* PDO300 were generated and followed by individual and combinatorial *in trans* complementation using relevant genes including *alg8/alg44* (encoding alginate-polymerizing proteins), *algX/algX(S269A)* (encoding alginate-acetylating/non-acetylating AlgX) and *algG/algG(D324A)* (encoding alginate-epimerizing/non-epimerizing AlgG). Generated knockout mutants lost mucoidity while mucoidity was restored upon *in trans* complementation with relevant genes. In order to shed light on the functional interaction between alginate-polymerizing and modifying subunits, the polymerisation degree, epimerisation degree and acetylation level of resulting alginates were assessed. The composition and the molecular weights of the respective alginates are summarized in Table 1. Fig. 5A shows in descending order the values obtained in regard to epimerisation, acetylation and polymerisation degree. ¹H-NMR spectra of compositional analysis of alginates are shown in Fig. S2 in the supplemental material. In order to investigate whether Alg8 and Alg44 are directly involved in polymannuronate synthesis, additional copies of both Alg8 and its interacting partner Alg44 were introduced into respective mutant backgrounds. Additional copies of Alg8 and/or Alg44 had a similar effect on alginate production such as resulting in high molecular weights with reduced epimerisation and acetylation when compared to the wild-type control (Fig, 5A; Table 1). The same effect of Alg8 and Alg44 on alginate polymerisation supported the hypothesis that they are subunits that together constitute the alginate polymerase.

AlgF, AlgI and AlgJ were proposed to form a protein complex constituting the alginate acetyltransferase/acetylerase (23, 70, 272). Recently, AlgX was demonstrated to play an independent role in alginate acetylation. AlgX is a two-domain protein including a domain with acetyltransferase activity and a carbohydrate-binding domain. Replacement of amino acid residues S269-H176-D174 which were proposed to

constitute the catalytic site resulted in non-acetylated alginate (24). AlgG, the epimerase, contains a conserved DPHD motif (residues 324-327) (16, 21, 26, 239), the proposed active site involved in epimerisation. Replacement of amino acid residues in this motif was shown to result in non-epimerized alginate while modified AlgG retained its protective role on nascent alginate against degradation in the periplasm. Here we used catalytically inactive variants of AlgX (S269A) and AlgG (D324A). When only the inactive AlgX variant was present, the resulting alginate was non-acetylated. Interestingly, additional copies of active AlgX or inactive AlgX resulted in the highest epimerisation values of $F_G = 0.36$. Additional copies of both AlgG and AlgX or inactive variants respectively, increased the degree of epimerisation of the resulting alginate (Fig. 5A). Additional copies of both AlgX and AlgG increased the degree of acetylation when compared to additional copies of only AlgX (Fig. 5A). Interestingly, additional copies of Alg44 enhanced acetylation 2.7-fold when compared to AlgX.

The correlation between the molecular weight of alginate and alginate modification, such as acetylation and epimerisation, was assessed (Fig. 5B). The molecular weight of the various alginates was determined by SEC-MALLS (Table 1; see Fig. S3 in the supplemental material). The highest molecular weight ($4653 \pm 1.1\%$ kDa corresponding to about 22876 uronic acid residues) was detected in alginates from strains with additional copies of the catalytically inactive epimerase variant AlgG (D324A). This was about a 70% increase in molecular weight when compared to alginate produced from strains with additional copies of epimerizing AlgG. The lowest molecular weight alginates were produced by strains harboring additional copies of those subunits contributing the highest levels of epimerisation i.e. AlgX ($F_G = 0.36$), AlgX (S269A) ($F_G = 0.36$) along with the lowest levels of acetylation (9.8% and 0). Non-acetylated and non-epimerized alginates ($F_G = 0$, Ac. = 0%) showed the lowest molecular weight ($1811 \pm 0.9\%$ kDa). Since $^1\text{H-NMR}$ spectra of the various alginates did not provide evidence for double bonds (between the C4 and C5 carbons leading to 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid), which are introduced by alginate lyase mediated degradation, the alginate lyase presumably did not influence the polymerisation degree. Additional copies of Alg8 and Alg44 gave rise to increased molecular weights ranging 3000 to 3800 kDa supporting their direct involvement in alginate chain synthesis. These data suggested that the alginate molecular weight is inversely correlated with alginate epimerisation but positively correlated with

acetylation (Fig. 5B). Alginates produced by the various strains showed a narrow molecular weight distribution with a polydispersity index close to 1 (Table 1).

***In vivo* alginate polymerase activity**

Alginate produced by the various strains was isolated and quantified (Fig. 5A and Table 1). Although additional copies of the various proteins increased the amount of alginate produced when compared to the reference strain PDO300 (pBBR1MCS-5), a significant variation of alginate productivity, i.e. alginate polymerase activity, was detected. Interestingly, additional copies of non-acetylating AlgX (S269A) and native AlgX mediated production of the largest amounts of alginate while epimerizing and non-epimerizing AlgG mediated the lowest level of production (Fig. 5A). Pairwise comparison of these four strains showed that more alginate is produced in the absence of modification events. The enhancing role of AlgX in alginate production was further supported when additional copies of AlgX together with AlgG led to a strong production of alginate. However, the non-acetylating and non-epimerizing pair of them resulted in a much lower quantity (Fig. 5A). Furthermore, all attempts to restore alginate production in PDO300 Δ *alg44* Δ *algX* with pBBR1MCS-5:*alg44:algX* failed. The mucoid phenotype of this double-gene knockout mutant was only restored when one of the introduced complementing genes, either *alg44* or *algX*, were integrated into the genome (*in cis* complementation using mini-CTX) and the other one presented *in trans* resulting in alginate production of 1.9 (g) / CDM (g).

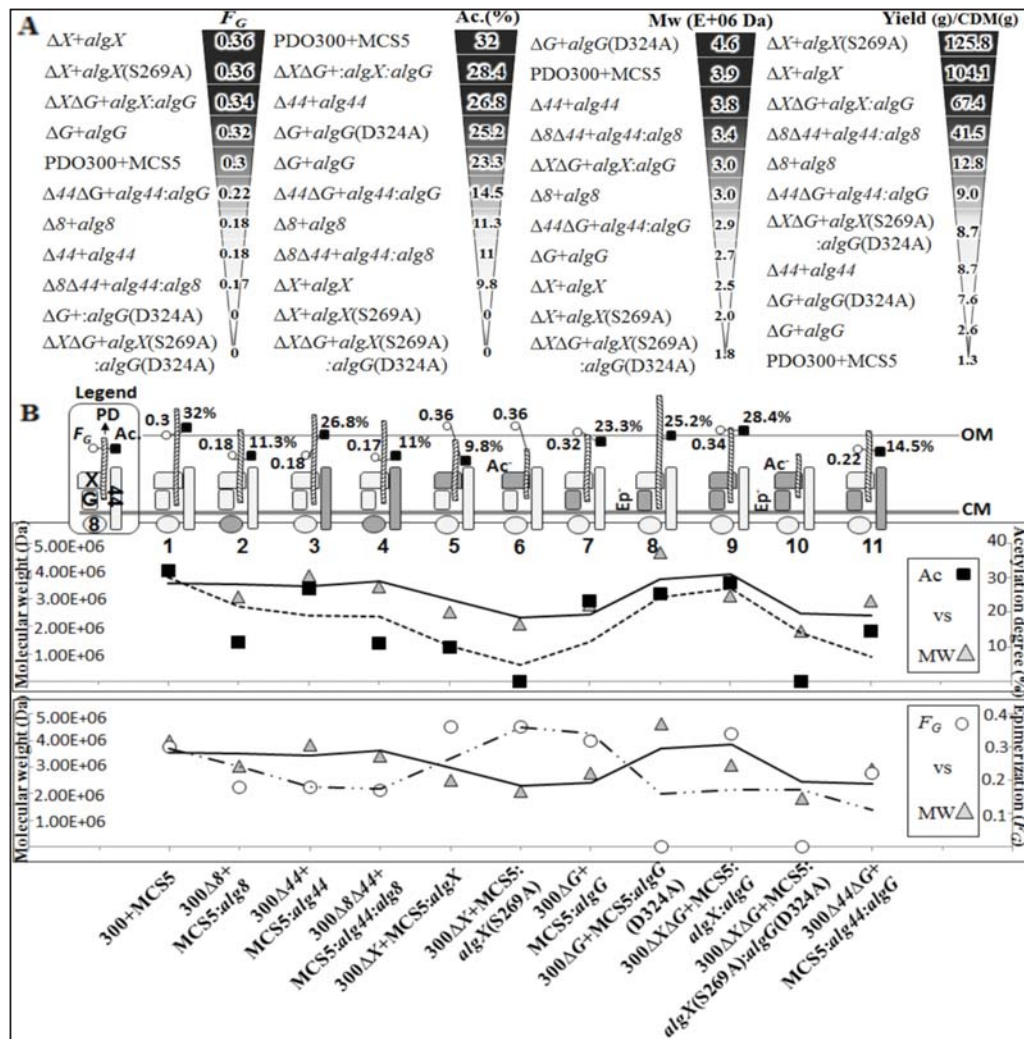


Fig. 5. Impact of putative alginate polymerase subunits on alginate polymerase activity, alginate polymerisation and composition and correlation between polymerisation and modification. (A) The values of molar fraction of G residue (F_G), acetylation degrees (Ac.%), mean molecular weights (Mw) and alginate yield are aligned with the strains producing the respective alginates. (B) Correlation between degree of acetylation, epimerisation and molecular weight of alginate. Presumable features (No. 1-11) show protein complexes constituted by Alg8, Alg44, AlgG and AlgX (cf. legend on top left corner of plot). The subunit produced upon *in trans* complementation is shown as darker shape(s). Inactive AlgX(S269A) and AlgG(D324A) are labelled as (Ac⁻) and (Ep⁻), respectively. The length of various alginates (PD) with respect to acetylation (Ac.%) and epimerisation (F_G) degrees are presented and proportionally illustrated for each feature. 300: PDO300; MCS5: pBBR1MCS-5; PD: polymerisation degree; OM: outer membrane; CM: cytoplasmic membrane.

TABLE 1 Composition and molecular weight analyses of alginate produced by different mutants

Mutant	F_G	F_M	F_{GAMG}	F_{MA}	Ac.%	\bar{M}_n (kDa)	\bar{M}_w (kDa)	PI	Alginate yield (g)/ CDM(g)
1 300 +MCS5	0.3	0.7	0.29	0.41	32	3927 ($\pm 0.864\%$)	3832 ($\pm 0.842\%$)	1.025 ($\pm 1.2\%$)	1.3 \pm 0.03
2 300 Δ 8- MCS5:alg8	0.18	0.82	0.17	0.65	11.3	3045 ($\pm 0.556\%$)	3037 ($\pm 0.551\%$)	1.003 ($\pm 0.7\%$)	12.8 \pm 1.03
3 300 Δ 44+ MCS5:alg44	0.18	0.82	0.18	0.64	26.8	3831 ($\pm 0.963\%$)	3650 ($\pm 0.950\%$)	1.05 ($\pm 1.3\%$)	8.7 \pm 0.53
4 300 Δ 44 Δ 8- MCS5:alg44:alg8	0.17	0.83	0.17	0.66	11	3369 ($\pm 0.839\%$)	3352 ($\pm 0.821\%$)	1.005 ($\pm 1.1\%$)	41.5 \pm 4.9
5 300 Δ X- MCS5:algX	0.36	0.64	0.36	0.28	9.8	2460 ($\pm 0.932\%$)	2447 ($\pm 0.913\%$)	1.005 ($\pm 1.3\%$)	104.1 \pm 5.5
6 300 Δ X+ MCS5:algX(S269A)	0.36	0.64	0.36	0.28	0	2086 ($\pm 0.960\%$)	2065 ($\pm 0.944\%$)	1.010 ($\pm 1.3\%$)	125.8 \pm 9.9
7 300 Δ G- MCS5:algG	0.32	0.68	0.32	0.36	23.3	2755 ($\pm 1.041\%$)	2726 ($\pm 0.986\%$)	1.011 ($\pm 1.4\%$)	2.6 \pm 0.04
8 300 Δ G+ MCS5:algG(D324A)	0	1	0	1	25.2	4653 ($\pm 1.097\%$)	4575 ($\pm 1.117\%$)	1.017 ($\pm 1.5\%$)	7.6 \pm 0.57
9 300 Δ X Δ G+ MCS5:algX:algG	0.34	0.66	0.34	0.32	28.4	3076 ($\pm 1.051\%$)	3044 ($\pm 1.029\%$)	1.011 ($\pm 1.4\%$)	67.42 \pm 4.8
10 300 Δ X Δ G+ MCS5:algX(S269A): algG(D324A)	0	1	0	1	0	1811 ($\pm 0.884\%$)	1716 ($\pm 0.888\%$)	1.055 ($\pm 1.2\%$)	8.7 \pm 0.42
11 300 Δ 44 Δ G+ MCS5:alg44-:algG	0.22	0.78	0.22	0.56	14.5	2907 ($\pm 0.966\%$)	2861 ($\pm 0.944\%$)	1.016 ($\pm 1.3\%$)	9.0 \pm 0.3

300:PDO300; MCS5: pBBR1MCS5-5; F_G : molar fraction of guluronate (G) residue; F_M : molar fraction of mannuronate (M) residue; F_{GAMG} : molar fraction of two consecutive G and M residues; F_{MA} : molar fraction of two consecutive M residues; Ac.: acetylation; \bar{M}_n : weight-average molecular weights; \bar{M}_w : number-average molecular weights; PI: polydispersity index; CDM: cell dry mass.

Microrheological analysis of various alginates

Particle-tracking microrheology was applied to assess the viscoelastic properties of the various resulting alginates. All alginates showed viscoelastic properties in which the solid-like elastic modulus G' was greater than the liquid-like viscous modulus G'' ($G' > G''$). The plot of particles mean square displacement (MSD) versus correlation time showed MSD curves of the alginates are distributed in four distinct categories (see Fig. S4 in the supplemental material). In the first category, the alginates produced from PDO300 Δ algG (pBBR1MCS-5:algG(D324A)) and PDO300 Δ alg8 (pBBR1MCS-5:alg8), respectively, without G-residues and with the highest molar fraction of MM-blocks, and both with very high molecular weight, showed the highest and quite similar viscoelastic properties ($G' = 0.41$, $G'' = 0.3$; $G' = 0.40$, $G'' = 0.28$, respectively). Interestingly, the alginates from PDO300 Δ alg44 (pBBR1MCS-5:alg44), PDO300 Δ algG (pBBR1MCS-5:algG) and PDO300 (pBBR1MCS-5) dropped into the second category with lower viscoelastic property. In the third category, showing lower viscoelastic properties, those alginates with molecular weight of ≤ 2000 kDa produced by PDO300 Δ algX (pBBR1MCS-5:algX(S269A)) and PDO300 Δ algX Δ algG (pBBR1MCS-5:algX(S269A): algG(D324A)) were found. Surprisingly, acetylated alginate from PDO300 Δ algX (pBBR1MCS-5:algX) was the only member of fourth category with the lowest viscoelastic property among all analysed samples. These results suggested that viscoelasticity was positively impacted by the molecular weight combined with high M content, while the presence of G residues and acetyl groups in the alginate chain lowered viscoelasticity. All these polymers showed greater elasticity than viscosity ($G' > G''$).

The impact of various alginates on biofilm formation

P. aeruginosa is capable of different modes of motility such as twitching, swarming and swimming, which are controlled by various regulatory pathways and environmental factors. These play an important role in biofilm formation and dispersal. Here, motility assays were conducted with strains capable of producing different alginates in order to assess the relationship between alginate composition/molecular weight, i.e. material properties, and motility, ultimately impacting on biofilm formation (see Fig. S5 in the supplemental material). All strains with alginate production greater than PDO300 (pBBR1MCS-5) showed lower twitching motility, while non-alginate producing knockout mutants showed greater twitching values. The lowest twitching motility

among all strains was found for PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*). Twitching motility differences between PDO300 Δ *algX* (pBBR1MCS-5:*algX*) and PDO300 Δ *algX* (pBBR1MCS-5:*algX*(S269A)) or between PDO300 Δ *algG* (pBBR1MCS-5:*algG*) and PDO300 Δ *algG* (pBBR1MCS-5:*algG*(D324A)) were insignificant (see Fig. S5 in the supplemental material).

Swarming motility, which occurs on semi-solid surfaces and is regulated by quorum sensing, was assessed as being lower in alginate producing strains than in their respective knockout mutants, except for PDO300 Δ *alg8* (pBBR1MCS-5:*alg8*) which showed slightly greater swarming motility than PDO300 Δ *alg8* (pBBR1MCS-5). Among the alginate-producing strains, the greatest value of swarming motility was found for strains PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*) (11.33 mm) and PDO300 Δ *algX* (pBBR1MCS-5:*algX*) (11.0 mm). PDO300 Δ *algG* (pBBR1MCS-5:*algG*(D324A)) (6.83 mm) showed slightly greater swarming motility than PDO300 Δ *algG* (pBBR1MCS-5:*algG*) (6.0 mm).

Swimming which occurs in aqueous environments was tested and the values obtained varied significantly among the strains. Generally, alginate producing strains showed lower swimming capability than knockout mutants except for the mutants PDO300 Δ *alg8* (pBBR1MCS-5:*alg8*) and PDO300 Δ *alg8* (pBBR1MCS-5) (see Fig. S5 in the supplemental material).

Confocal laser scanning microscopy images of biofilms formed by mutant PDO300 Δ *algX* (pBBR1MCS-5:*algX*) and its non-acetylating counterpart, PDO300 Δ *algX* (pBBR1MCS-5:*algX*(S269A)) highlighted the crucial role of acetylation of alginate for developing biofilms and cellular arrangements (Fig. 6). Comparison of the two strains revealed significant differences in elevated structures and the distribution of microcolonies. For example, the structures formed by strains producing acetylated alginates were perfectly shaped and developed with a biovolume of $5.5 \pm 1.26 \mu\text{m}^3 \mu\text{m}^{-2}$ and a maximum height of 83 μm , while those formed with non-acetylated alginate showed a smaller biovolume of $3.9 \pm 0.2 \mu\text{m}^3 \mu\text{m}^{-2}$ and a reduced height of 26 μm , with irregular architecture. Interestingly, the strain producing acetylated alginate did not produce a multicellular base-layer and cells were organized in pillar-shaped architectures similar to structures described for the architectures of Psl-overproducing strain (i.e. *P. aeruginosa* WFPA801) (273, 274). In contrast, the strain producing non-acetylated alginate formed a biofilm with disordered and scattered microcolonies (Fig. 6, frame 3). Furthermore, PDO300 Δ *algX* (pBBR1MCS-5:*algX*) formed a biofilm with

1.5-fold more compactness and 31% more live cells than the biofilm formed by PDO300 Δ *algX*(pBBR1MCS-5:*algX*(S269A)) (Table 2). PDO300 Δ *algX*(pBBR1MCS-5) mutant did not form a structured biofilm (Fig. 6, frame 4) but a multicellular layer with a thickness of 6 μ m.

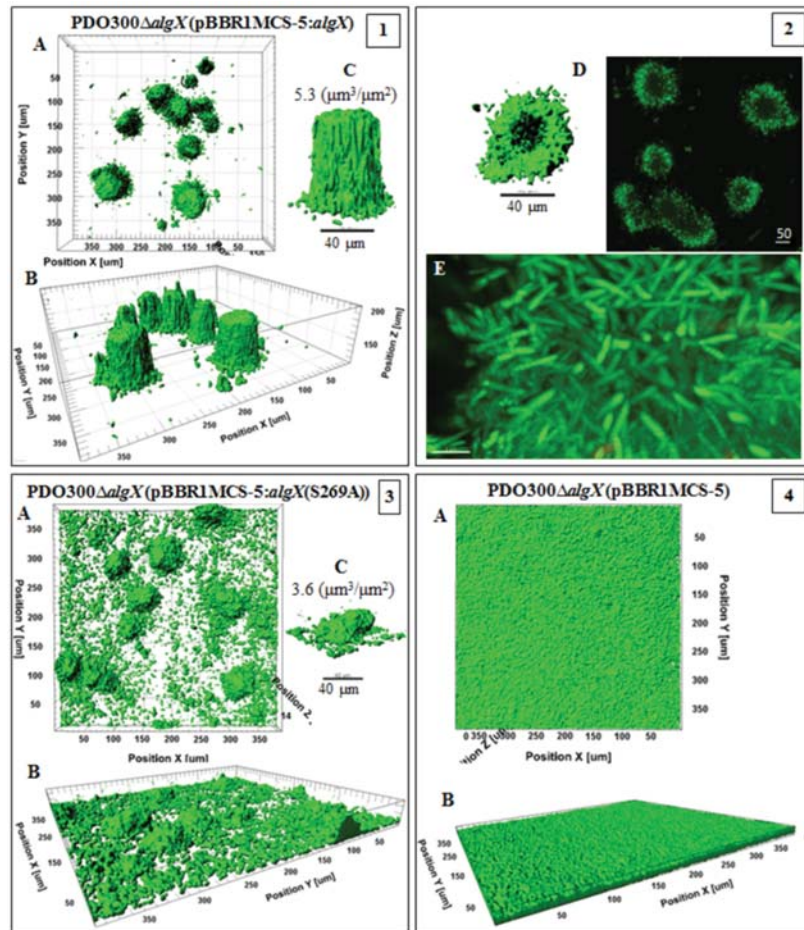


Fig. 6. Biofilm architecture of mutants producing acetylated and non-acetylated alginates. This figure shows biofilm formation and architecture of the mutants PDO300 Δ *algX*(pBBR1MCS-5:*algX*) (frames 1, 2), PDO300 Δ *algX*(pBBR1MCS-5:*algX*(S269A)) (frame 3) which produce acetylated and non-acetylated alginates, respectively, and PDO300 Δ *algX*(pBBR1MCS-5) (frame 4) with no alginate production. In all frames A-C shows top view, side views and a representative of typical highly structured cell community, respectively. The cell community dimensions are provided as μ m³ μ m⁻².

PDO300 Δ *algG* (pBBR1MCS-5:*algG*(D324A)) which produced a high molecular-weight, acetylated polymannuronate with strong viscoelasticity acquired the largest biovolume of $6.0 \pm 0.22 \mu$ m³ μ m⁻² (Fig. 7, frame 3). Interestingly, adjacent structures were networked with horizontal appendages, with void spaces and channels formed

underneath whole structures, likely to constitute water channels (Fig. 7, frame 4). On the other hand, PDO300 Δ *algG* (pBBR1MCS-5:*algG*) which produced lower molecular weight acetylated and G residue-containing alginate formed elevated, but less developed structures with less biovolume of $4.8 \pm 0.22 \mu\text{m}^3 \mu\text{m}^{-2}$ (Fig.7, frame 1). The base layers formed by both strains were dense and covered the whole area of the surface. The biofilm of PDO300 Δ *algG* (pBBR1MCS-5) was a homogenous layer of cells ($7\mu\text{m}$ thickness) without elevated structures (Fig. 7, frame 2).

PDO300 Δ *algG* Δ *algX* (pBBR1MCS-5: *algX*(S269A):*algG*(D324A)) produced a non-acetylated polymannuronate with a low molecular weight and the respective biofilm was composed of very long and narrowly elevated structures (Fig. 8, frame 1, 2). The biovolume was $1.5 \pm 0.2 \mu\text{m}^3 \mu\text{m}^{-2}$) which was less than for all the other investigated strains (Table 2).

Strains PDO300 Δ *alg8* (pBBR1MCS-5:*alg8*) and PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*) established heterogeneous highly structured biofilms (Fig. 9). The former formed a biovolume of $3.95 \pm 0.43 \mu\text{m}^3 \mu\text{m}^{-2}$ and compactness of $6.09\text{E}+02$ while the latter generated very dense and large structures with biovolume of $5.8 \pm 0.43 \mu\text{m}^3 \mu\text{m}^{-2}$ but less compactness ($4.43\text{E}+02$). Both mutants showed higher numbers of dead cells among all applied mutants but less than wild-type (Table 2). Conversely, PDO300 Δ *alg8* (pBBR1MCS-5) and PDO300 Δ *alg44* (pBBR1MCS-5) generated homogenous biofilm without elevated or highly structured architectures. Compactness values and dead/live ratios are summarized in Table 2.

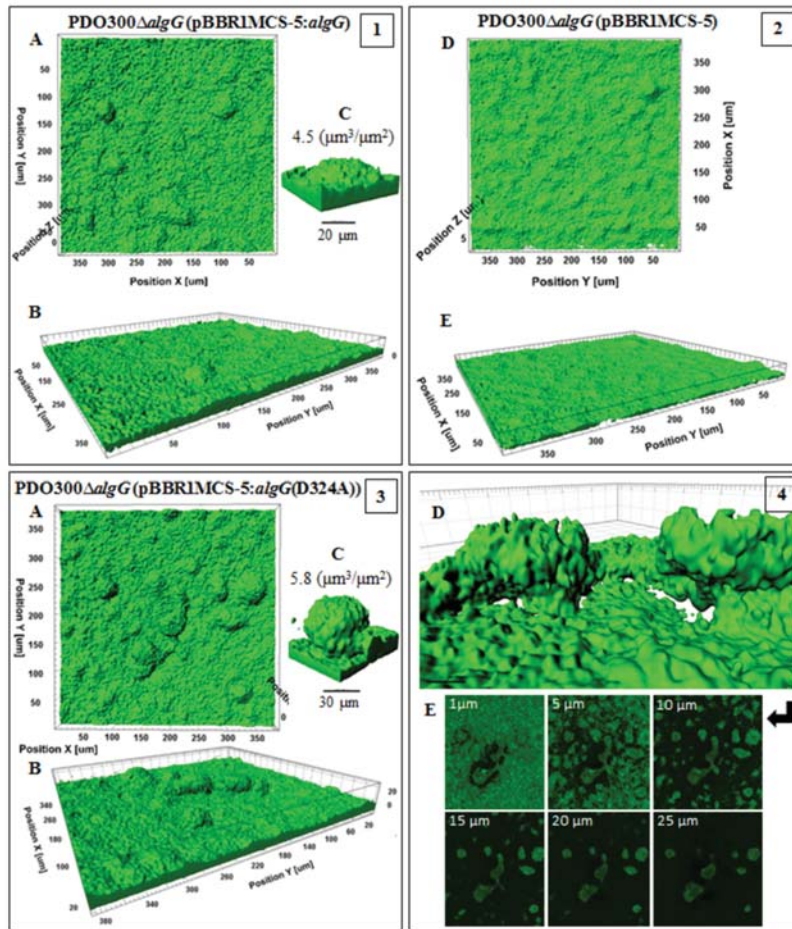


Fig. 7. Biofilm architecture of mutants producing epimerized and non-epimerized alginates. This figure shows biofilm formation and architecture of the mutants PDO300 Δ algG(pBBR1MCS-5:algG) (frame 1), PDO300 Δ algG(pBBR1MCS-5:algG(D324A)) (frames 3 and 4) which produce, respectively, epimerized (poly-MG) and non-epimerized (poly-M) alginates and PDO300 Δ algG(pBBR1MCS-5) (frame 2) with no alginate production. In all frames A, B and C shows respectively top view, side views and a representative of typical highly structured cell communities for that mutant with biovolume per area ($\mu\text{m}^3 \mu\text{m}^{-2}$) ratio. In frame 3, poly-M alginate-based biofilm is highly developed than poly-MG alginate-based in frame 1, presenting larger biovolume and biovolume per area ratio. Cells of both mutant covered entire cover slide surface. Frame 4D and E represent the architecture of poly-M alginate-based microcolonies in which two adjacent structures are connected with horizontal appendages and free-cell void cavities channeled underneath of microcolonies. Frame 4E shows 6 different slices of microcolonies with connected structure at the middle of figures surrounded by free-cell and matrix areas. Frame 2 represents homogenous cell community of non-mucoid mutant without highly structured architecture.

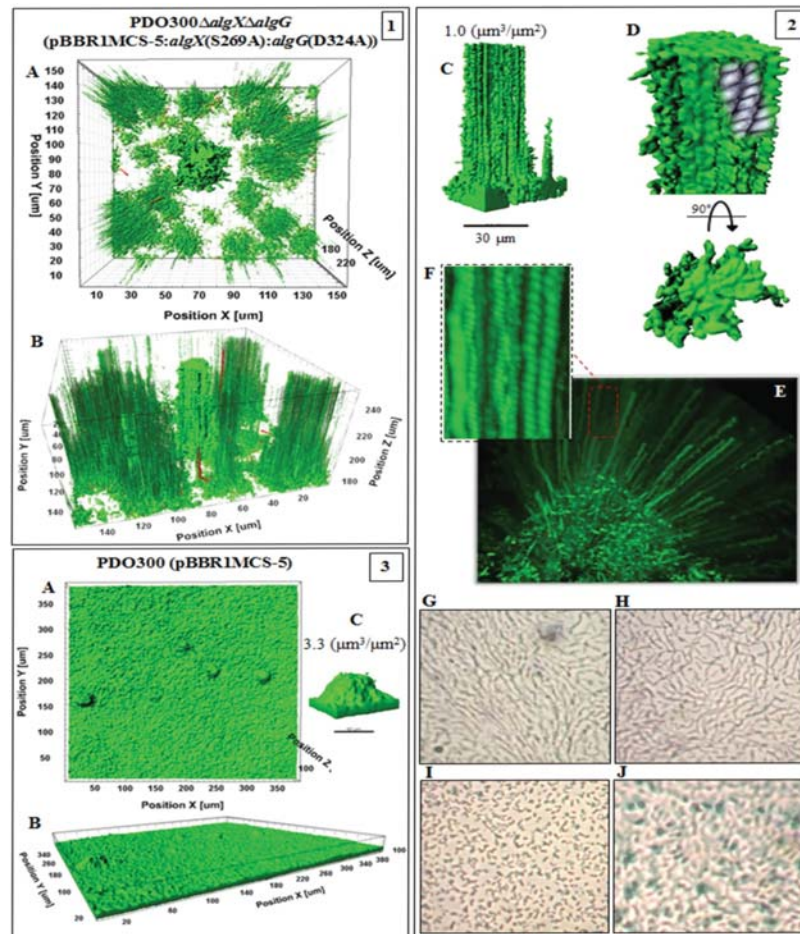


Fig. 8. Biofilm architecture of mutant producing non-epimerized and non-acetylated alginates and wild-type. This figure shows biofilm formation and architecture of the mutants PDO300 Δ algX Δ algG(pBBR1MCS-5:algX(S269A):algG(D324A)) (frames 1, 2) and PDO300 (pBBR1MCS-5) (frame 3). In all frames A, B and C shows respectively top view, side views and a representative of typical highly structured cell communities for that mutant with biovolume per area ($\mu\text{m}^3 \mu\text{m}^{-2}$) ratio. Biofilm architecture visualized for mutant producing non-acetylated poly-M alginate (frames 1, 2) was remarkably different from other applied mutants. Affected by alginate properties, emerging biofilm consists of very narrow but long elevated microcolonies representing longitudinal cell trails or strips indicating stigmergic self-organization and adaptation of cells in weak matrices. Frame 2D-F represents close side and top views of one of the microcolonies and cell trails and cell-cell interactions in each cell trail are depicted in sketches. Frame 2 G-J shows micrographs (40X magnification) of the edge (H, I) and surface (G, J) of mucoid colonies of PDO300 Δ algX Δ algG(pBBR1MCS-5:algX(S269A):algG(D324A)) (G, H) and PDO300 (pBBR1MCS-5) (I, J) forming a thin layer on PIA medium after incubation at 37°C for 18 h. Organization of cells of PDO300 Δ algX Δ algG(pBBR1MCS-5:algX(S269A):algG(D324A)) mutant showed linear filamentous aggregation pattern. Wild-type biofilm architecture is presented in frame 3.

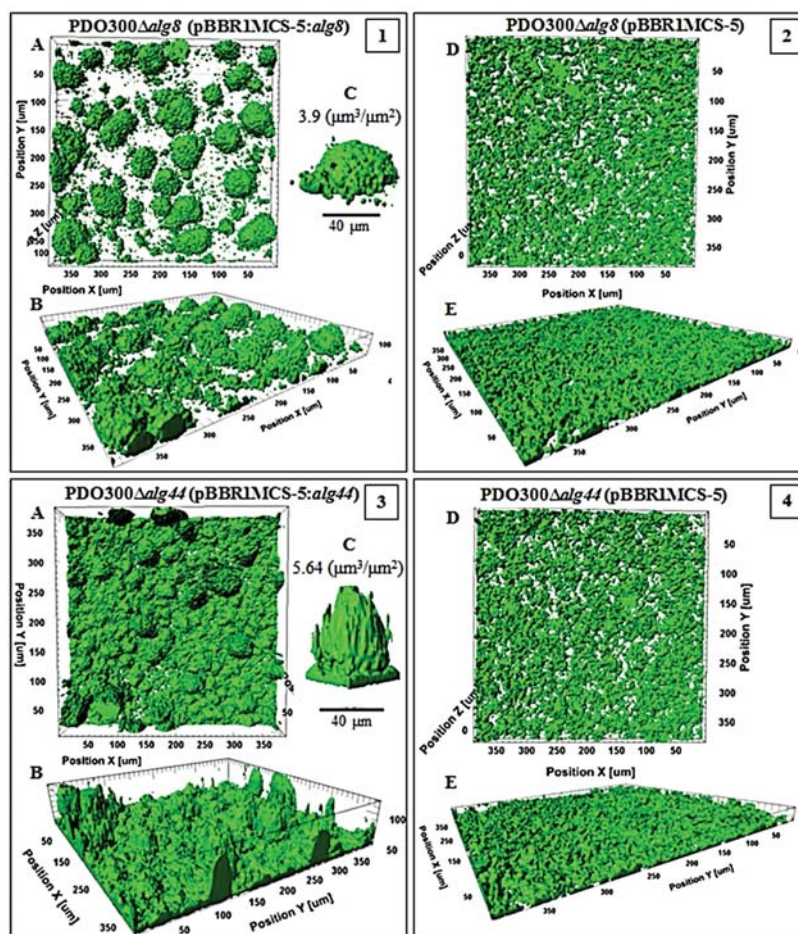


Fig. 9. Biofilm architecture of mutant producing high mannuronate molar fraction and M-block. This figure shows biofilm formation and architecture of the mutants PDO300Δalg8 (pBBR1MCS-5:alg8) (frame 1) and PDO300Δalg44 (pBBR1MCS-5:alg44) (frame 3) and non-mucoid mutants (frames 2, 4). In all frames A-C shows respectively top view, side views and a representative of typical highly structured cell communities for that mutant with biovolume per area ($\mu\text{m}^3 \mu\text{m}^{-2}$) ratio. Both mutants produce alginates with highest degree of M-block occurrence but very different degree of acetylation. PDO300Δalg44 (pBBR1MCS-5:alg44) (frame 3) which produce highly acetylated alginate established a very dense and highly developed and larger microcolonies than PDO300Δalg8 (pBBR1MCS-5:alg8) (frame 1). One explanation for this significant difference is the presence of additional copy of Alg44 which senses c-di-GMP which is a common secondary messenger in the cells governing physiological condition of cells during colonization. However, non-mucoid mutants did not establish highly structured biofilm and microcolonies.

Table 2. Compactness and dead/live ratio calculated for analysed biofilms

mutant	Compactness*	Dead/live ratio**
300Δ8+MCS5: <i>alg8</i>	6.09E+02	1.43±0.10
300Δ44+ MCS5: <i>alg44</i>	4.43E+02	1.17±0.04
300ΔG+ MCS5: <i>algG</i>	2.77E+02	0.70±0.06
300ΔG+ MCS5: <i>algG</i> (D324A)	1.68E+02	0.97±0.04
300ΔX+ MCS5: <i>algX</i>	3.00E+02	0.42±0.02
300ΔX+ MCS5: <i>algX</i> (S269A)	2.20E+02	0.55±0.02
300ΔGΔX+MCS5: <i>algG</i> (D324A): <i>algX</i> (S269A)	1.20E+03	0.49±0.03
300+MCS5	4.46E+02	2.40±0.29
300Δ8+MCS5	1.39E+02	1.46
300Δ44+MCS5	1.42E+02	0.7
300ΔG+MCS5	8.90E+01	1.01
300ΔX+MCS5	1.16E+02	0.94

300:PDO300; MCS5: pBBR1MCS-5; *Total fluorescence per volume of biofilm;

**Ratio between red and green fluorescence shown by each biofilm-forming mutant

Discussion

In this study, we investigated the relationship between alginate polymerisation and modification, the functional role of the subunits Alg8, Alg44, AlgG and AlgX and their physical and functional interaction. A range of alginate compositions and molecular weights exhibiting various material properties were produced by engineered *P. aeruginosa* strains and their impact on motility and biofilms formation was assessed. This study revealed protein-protein interaction between Alg8 and Alg44 respectively proposed as alginate polymerase and co-polymerase (Fig. 1). Alg44 was found to interact with AlgK (Fig. 2A) which is an outer membrane lipoprotein that aids correct localization of the AlgE porin to the outer membrane. This study provides experimental evidence for the previously suggested presence of an Alg8-Alg44-AlgX-AlgK-AlgE multiprotein complex bridging cell envelope forming the alginate polymerisation/modification/secretion machinery (80). The absence of the previously observed AlgK-X interaction after DSG cross-linking and anti-AlgX immunoblotting (Fig. 2D) was presumable due to a lack of suitable cross-linking sites (K residues 7Å apart of each other) (79). In addition, immunoblotting/cross-linking data suggested that Alg44 forms a dimer. However, this dimer was not observed when Alg44 was overproduced from a plasmid indicating that Alg44 dimerisation might be susceptible to changes in stoichiometry (Fig. 2C, D). Bioinformatics analysis of the periplasmic part of Alg44 suggests the presence of coil-coiled structure (Coil/Pcoils-based score: 0.4) which have been described for membrane fusion proteins (MFPs) such as MexA and

polysaccharide co-polymerases (PCPs) to contribute to oligomerization (275). Here it was demonstrated that Alg44 localization, stability and protein-protein interaction were not impacted by altering c-di-GMP level or by non-c-di-GMP binding variants (Fig. 3; see also Fig. S1). Our results indicated that the c-di-GMP mediated activation mechanism of alginate polymerisation differs from the activation mechanism of cellulose synthase in which c-di-GMP releases an autoinhibited state by breaking a salt bridge (270). However residue H323 of Alg8 might be involved in c-di-GMP-dependent activation of alginate polymerase as reduced c-di-GMP levels did not impair *in vivo* Alg8 activity (Fig. 4).

The first experimental evidence was obtained that AlgX and AlgG exhibit a mutually auxiliary behavior, suggesting that the two modification events (acetylation, epimerisation) are not competitive, but linked (26). In addition, we propose a new auxiliary role for Alg44 in acetylation besides being necessary for c-di-GMP dependent activation of alginate polymerisation (Table 1; Fig. 5A). Failed attempts in complementing PDO300 Δ alg44 Δ algX with pBBR1MCS-5:alg44:algX, but successful complementation if one gene was present *in cis* and the other *in trans* suggested that the stoichiometry of these two proteins is critical for proper performance of the multiprotein complex.

This study revealed how alginate polymerisation (Alg8, Alg44) is aligned with alginate modification (AlgG, AlgX). As shown in Fig. 5A, additional copies of active or inactive AlgX acetyltransferase significantly increased the molar fraction of G residues as well as productivity which appeared inversely correlated with the alginate molecular weight (Fig. 5A; Table 1). This suggests a new role of AlgX in epimerisation and as periplasmic scaffold protein playing a key role in efficient translocation of the alginate chain across the periplasm. Recently, it was reported that AlgX binds to polymannuronic acid in a length-dependent manner and acts as terminal acetyltransferase (22).

Interestingly, restoration of alginate production of the AlgG negative mutant by an inactive variant of AlgG led to a significantly increased alginate molecular weight when compared to active AlgG suggesting that AlgG as scaffold subunit is critical for processivity of alginate polymerisation while the actual epimerisation event interferes with processivity (Fig. 5A, B). Furthermore, the role of AlgG mediated epimerisation on alginate length might be due to AlgG mediated alginate degradation as polysaccharide epimerases show a similar reaction mechanism compared to

polysaccharide lyases (26). This finding might also explain why algal alginates with high molar fraction of G residues introduced by epimerases have very low molecular weights (110). Acetylation was correlated with the molecular weight suggesting that there was no negative impact on processivity of alginate polymerisation (Fig. 5B).

In general, additional copies of any subunit increased alginate production when compared to the reference strain, indicating that the stoichiometry of the various subunits is less critical for the activity of the multiprotein complex (Table 1). Based on these results with regards to the roles of the investigated subunits in alginate synthesis and modification, a revised model of the alginate biosynthesis multiprotein complex was proposed (Fig. 10).

To shed light on structure-function relationship of the various alginates, their viscoelasticity was assessed. The presence of acetyl groups lowered viscoelasticity by possibly interfering with intermolecular alginate chain interactions (see Fig. S4 in the supplemental material). In contrast, increasing molar fractions of MM-blocks and higher molecular weights increased viscoelasticity (see Fig. S4 in the supplemental material). Acetylated alginates gave rise to well-developed and highly organized heterogeneous architectures and promoted cell aggregations (Fig. 6) which was consistent with previous studies (276) but these findings suggested viscoelasticity is not critical for biofilm architecture formation. Fig. 7 shows that non-epimerized alginate (polymannuronate) with high molecular weight and the strong viscoelasticity supported the establishment of these biofilm features and that by controlling the molar fraction of G residues biofilm architecture characteristics could be adapted to various environments. We showed that the lack of G-residues and acetyl groups caused the formation of undeveloped and narrow microcolonies which were supported by specific long trails or strips of cells emerging from stigmergic self-organization of cells affected by this particular alginate (Fig. 8) (277). This was further evidence for the role of alginate material properties on the formation of particular biofilm architectures and cellular aggregation patterns.

Motility mediated by twitching, swarming and swimming was assessed using strains producing various alginates. These motilities are critical for biofilm development and dispersal (267). Here, we showed overproduction of alginate interferes with motility. Previously, it was shown that high levels of c-di-GMP reduced motility while increasing production of various exopolysaccharides and biofilm formation (278). Moreover, our results showed these motilities are independent of the acetyl group and G residue

content of the respective alginate (Fig. S5 in the supplemental material). Overall, this study led to updating protein-protein interaction network constituting proposed alginate polymerisation/modification/secretion multiprotein complex in *P. aeruginosa* and the development of strains producing a range of alginates enabling the analysis of structure-function relationships, from a materials property and biological function perspective such as demonstrating that viscoelasticity of alginate contributed to enhanced cell aggregation during biofilm formation.

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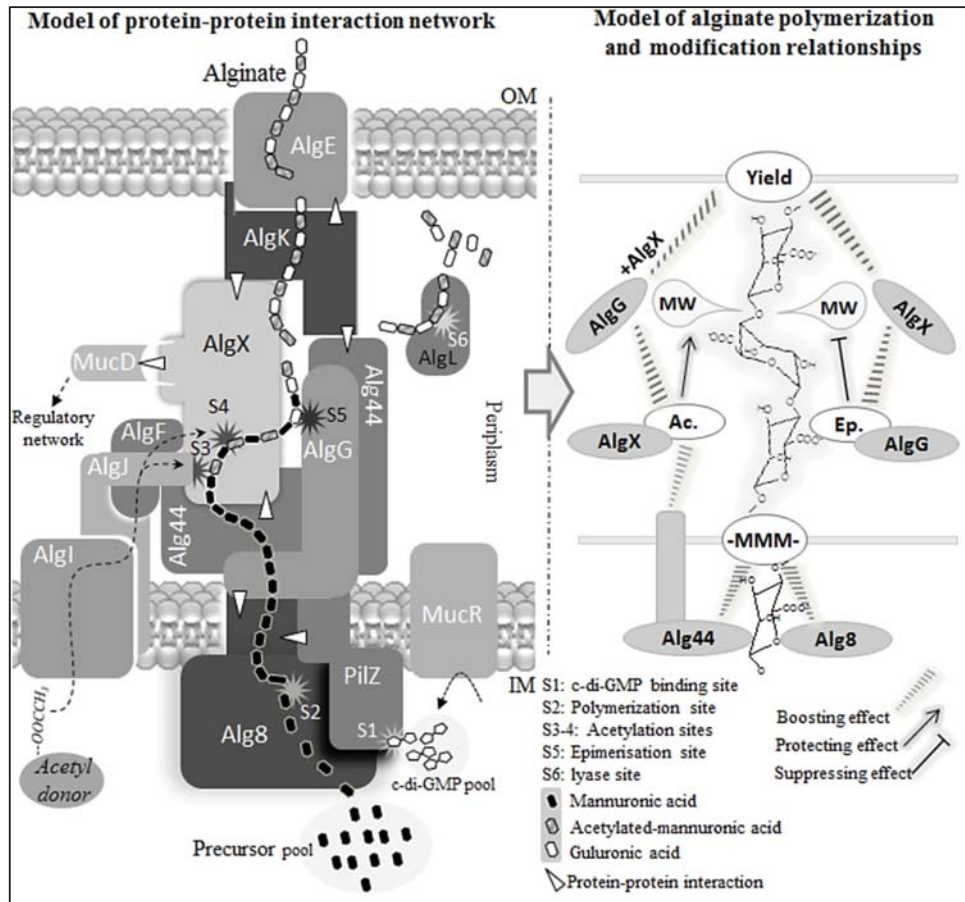


FIG 10 A new proposed model of alginate biosynthesis machinery complex and interactive performances of protein functionality over alginate polymerisation, acetylation, epimerisation and length determination. Based on experimental results obtained in the current study, functional and physical interactions of subunits (left side of figure) were modeled into a multiprotein complex. In this model different binary interactions are marked with white triangles. Our finding of Alg8-Alg44 and Alg44-AlgK interactions together with previously discovered interaction of AlgK-AlgE (80) constitute the periplasmic part of the multiprotein complex connecting the cytoplasmic membrane with the outer membrane. The model also includes the following interactions Alg44-AlgX, Alg8-AlgG, MucD-AlgX-AlgK (79, 80, 279) in support of the periplasmic scaffold guiding nascent alginate for translocation and secretion. This model shows alginate production is positively regulated by c-di-GMP binding to Alg44 interacting (S1) targeting catalytic site of Alg8 polymerase (S2) through an unknown mechanism. Then translocation across periplasmic scaffold is coupled with interactive functional performances of modification events and proteins (right side of model) for alginate length regulation and a series of associated modification events (S3-5). AlgL is responsible for degrading misguided alginate accumulating in periplasm (S6). MucD protein links the complex with the posttranslational alginate regulatory network via an interaction with AlgX.

Supplemental Materials

Materials and Methods

Bacterial strains, plasmids, growth conditions and chemicals

Strains and plasmids used in this study are summarized in Table S1. *P. aeruginosa* and *Escherichia coli* strains were cultivated in Luria Broth medium supplemented by appropriate antibiotics and were grown at 37°C. For those assays designed for studying alginate production, compositional and molecular mass analyses, bacterial phenotype and protein-protein interaction and stability, Difco™ Pseudomonas Isolation Agar (PIA) medium was used which was supplemented by appropriate antibiotics. To rule out the effect of growth condition and media on alginate composition and quantity, all experiments were conducted under the same conditions and using the same batch culture at the same time. All chemicals were purchased from Sigma-Aldrich and Merck KGaA unless otherwise mentioned. All applied enzymes used for cloning were manufactured by Roche, New England Biolabs GmbH or Invitrogen.

Construction of isogenic single- and double-gene knockout mutant in *alg8*, *alg44*, *algG* and *algX* genes

Marker-free single- and double-gene knockout mutants PDO300 Δ *alg8* Δ *alg44*, PDO300 Δ *algG*, PDO300 Δ *algX* Δ *algG*, PDO300 Δ *alg44* Δ *algG* were generated through two events of homologous recombination using suicide plasmid pEX100T Δ *alg44* Ω Gm, pEX100T Δ *alg8* Ω Gm, pEX100T Δ *algG* Ω Gm and pEX100T Δ *algX* Ω Gm. Strains PDO300 Δ *alg8* and PDO300 Δ *alg44* were generated previously (268). These suicide plasmids containing knockout genes (only 5' and 3' flanking regions of the respective gene) which were disrupted by the *aacCI* gene (1,100-bp fragment encoding gentamicin acetyltransferase) flanked by two *FRT* sites (flippase recombinase target) (11). They were individually transferred into *E. coli* S17-1 competent cells as donor for transfer into PDO300, PDO300 Δ *alg44* and PDO300 Δ *algX*. Transconjugants were selected on mineral salt medium (MSM) containing 100 μ g ml⁻¹ gentamicin and 5% (wt/vol) sucrose (245). Cells emerging from double-crossover events grew on this medium and those cells harboring suicide plasmid with counter-selectable marker, *sacB*, or undergoing single crossover events did not grow. Gene replacement was confirmed after subculture of cells on PIA medium containing 300 μ g/ml gentamicin followed by PCR with primers binding to sites outside the flanking regions of the respective target gene.

E. coli SM10 was used as donor to transfer the flippase recombinase encoding vector pFLP2 into presumable knockout mutants and after 24 h of cultivation on PIA medium containing 5% (wt/vol) sucrose, they were screened based on sensitivity to gentamicin and carbenicillin (242). Gentamicin and carbenicillin-sensitive cells were analysed by PCR with primers *alg8* up/down, *alg44* up/down, *algX* up/down and *algG* up/down for successful loss of the *FTR-aacCI-FRT* cassette and to confirm that the target gene was deleted.

***In trans*-complementation of single- and double-gene knockout mutants**

The genes *alg8*, *alg44*, *algG* and *algX* of *P. aeruginosa* PAO1 were individually amplified by PCR and separately ligated into pGEM-T Easy vector (Promega) for sequencing. The genes *algG*(D324A) and *algX*(S269A) respectively encoding point-mutated non-epimerising AlgG and non-acetylating AlgX proteins were synthesized by GenScript. These genes were individually or simultaneously ligated into the corresponding sites of pBBR1MCS-5 (246) (cf. Table S1) resulting in the final constructs pBBR1MCS-5:*alg44:alg8*, pBBR1MCS-5:*algG*, pBBR1MCS-5:*algX*, pBBR1MCS-5:*algX:algG*, pBBR1MCS-5:*alg44:algG*, pBBR1MCS-5:*algX*(S269A), pBBR1MCS-5:*algG*(D324A) and pBBR1MCS-5:*algX*(S269A):*algG*(D324A). These constructs were transferred into appropriate single- and double-gene knockout mutants via transconjugation using *E. coli* S17-1 as donor or electroporation. Resultant transformants were selected on PIA medium containing 300 µg ml⁻¹ gentamicin and confirmed by selecting cells with mucoid phenotypes followed by plasmid isolation and analysis. Phenotypic characterization of transformants included alginate isolation and quantification. All generated strains are listed in Table E3.

Chromosomal integration of *alg8-6his*, *alg44-6his*, *algG-6his* and *algX-6his* and *cis*-complementation of single-gene knockout mutants

In order to integrate genes encoding the respective hexahistidine (6his) tagged alginate protein into the genome, the plasmid mini-CTX-*lacZ* (248) was used and plasmids mini-CTX:*Palg8-6his*, mini-CTX:*Palg44-6his*, mini-CTX:*PalgG-6his* and mini-CTX:*PalgX-6his* were constructed as follows. The promoter region at -901 bp relative to the *algU* open reading frame was amplified using primers *palgUPstIF* and *palgUHindIIIIR* (247). The *alg8*, *alg44*, *algG* and *algX* regions were amplified and PCR products were ligated into pGEM-T Easy vector for sequence verification. The promoter region was

hydrolyzed with *Pst*I and *Hind*III and the genes were digested with *Hind*III and *Bam*HI followed by ligation of fragments into mini-CTX-*lacZ* hydrolyzed with *Pst*I and *Bam*HI, resulting in the generation of abovementioned plasmids. These plasmids each were electroporated into PDO300 Δ *alg8*, PDO300 Δ *alg44*, PDO300 Δ *algG* and PDO300 Δ *algX*. Transformants were selected on PIA containing 150 μ g ml⁻¹ tetracycline. Integration of promoter region and the genes into the genome was confirmed using PCR with primers PserUp and PserDown. To remove the mini-CTX-*lacZ* backbone plasmid pFLP2 was used as described above.

Site-specific mutations and deletions of *alg44* and *alg8*

Two highly conserved R residues of PilZ_{Alg44} domain's R17XXXR21 motif binding to secondary messenger c-di-GMP and the residues E322, H323 and E326 of Alg8 were mutated to D or A using either utilizing the QuikChange[®] II Site-Directed Mutagenesis Kit (Stratagene) or by DNA synthesis (GenScript). The resultant plasmids were pBBR1MCS-5:*alg44*(R17A), pBBR1MCS-5:*alg44*(R21A), pBBR1MCS-5:*alg44*(R21D)-6his, pBBR1MCS-5:*alg44*(R17A, R21A), pBBR1MCS-5:*alg8*(E322A), pBBR1MCS-5:*alg8*(H323A) and pBBR1MCS-5:*alg8*(E326A), pBBR1MCS-5:*alg44*(R17A):*alg8*(E322A), pBBR1MCS-5:*alg44*(R17A):*alg8*(H323A), pBBR1MCS-5:*alg44*(R17A):*alg8*(E326A), pBBR1MCS-5:*alg44*(R21A):*alg8*(E322A), pBBR1MCS-5:*alg44*(R21A):*alg8*(H323A), pBBR1MCS-5:*alg44*(R21A):*alg8*(E326A). Combinations of these mutated genes with *rocR* (PA3947) were also generated as mentioned in Table E3. Oligonucleotide deletion of PilZ_{Alg44} domain from amino acid 40 to 74 designated as (Δ 40-74aa_{PilZ}) and C-terminal truncation of a periplasmic part of Alg44 of 26 amino acids (Δ 364-389aa) were performed through the replacement of short synthesized DNA fragments containing these deletions yielding pBBR1MCS-5:*alg44*-6his(Δ 40-74aa_{PilZ}) and pBBR1MCS-5:*alg44*-6his(Δ 364-389aa). These plasmids were electroporated into PDO300 Δ *alg8*, PDO300 Δ *alg44* or PDO300 Δ *alg8* Δ *alg44* mutants generating PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*(R21D)-6his), PDO300 Δ *alg44*(pBBR1MCS-5:*alg44*-6his(Δ 40-74aa_{PilZ})), PDO300 Δ *alg44*(pBBR1MCS-5:*alg44*-6his(Δ 364-389aa)), PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*(R17A)), PDO300 Δ *alg44* (pBBR1MCS-5:*alg44*(R21A)), PDO300 Δ *alg8* (pBBR1MCS-5:*alg44*(E322A)), PDO300 Δ *alg8* (pBBR1MCS-5:*alg44*(H323A)), PDO300 Δ *alg8* (pBBR1MCS-5:*alg44*(E326A)), PDO300 Δ *alg8* Δ *alg44* (pBBR1MCS-5:*alg44*(R17A):*alg8*(E322A)),

PDO300 Δ alg8 Δ alg44 (pBBR1MCS-5:alg44(R17A):alg8(H323A)),
PDO300 Δ alg8 Δ alg44 (pBBR1MCS-5:alg44(R17A):alg8(E326A)),
PDO300 Δ alg8 Δ alg44 (pBBR1MCS-5:alg44(R21A):alg8(E322A)),
PDO300 Δ alg8 Δ alg44 (pBBR1MCS-5:alg44(R21A):alg8(H323A)),
PDO300 Δ alg8 Δ alg44 (pBBR1MCS-5:alg44(R21A):alg8(E326A)). Transformants were selected on PIA medium supplemented by 300 μ g ml⁻¹ gentamicin and confirmed by plasmid isolation and analysis.

***In vivo* detection of protein-protein interaction network**

Dependent on the applicability and the efficiency of protein-protein interaction methods for membrane-anchored proteins and those involved in the multi-protein complexes, three different methods including pull-down under native condition, *in vivo* chemical crosslinking and bacterial two-hybrid system (BACTH) were successfully applied. However different characteristics of proteins involved in alginate multi-protein complex were considered in designing these experiments and they are described as follows.

His pull-down assay under native conditions

For pulling down hexahistidine-tagged Alg44 (Alg44-6his), Alg8 (Alg8-6his) and defective variants of Alg44-6his under native condition, a His-spin protein miniprep kit (Zymo Research) was used. Cells grown on solid media were scraped-off and washed twice with saline solution and pelleted. Enzymatic cell lysis was carried out by adding lysis buffer prepared with buffer A (100 mM NaH₂PO₄, 100 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5% Triton, Roche complete protease inhibitor EDTA-free) containing 0.1 mg ml⁻¹ lysozyme and 0.1 mg ml⁻¹ DNaseI and incubated on ice for 30 min and subsequently lysed by sonication. Cell debris was removed by centrifugation at 15,000 \times g for 20 min at 4 °C. Subsequently, ultracentrifugation at 100,000 \times g was carried out for 1 h at 4 °C to isolate the envelope fraction and embedded proteins. Sediments were solubilized with buffer A containing 10 mM imidazole and mixed with the resin. Buffer A supplemented with 50 mM and 400 mM imidazole was respectively used for washing twice and elution. Eluates were used for protein analysis.

***In vivo* chemical cross-linking**

For Alg44-6his proteins *in vivo* chemical crosslinking was performed using 400 ml overnight liquid cultures as described previously (80). Briefly, disuccinimidyl glutarate (DSG, Pierce) was used for covalently linking primary amine moieties of two lysine residues in the vicinity of 7.7Å. Crosslinking reaction was carried out in 5 ml of phosphate-buffered saline (PBS) containing cells and 1.5 mM DSG (dissolved in dimethyl sulfoxide (DMSO)) at 37 °C for 30 min and terminated with 20 mM Tris-HCl (f. conc., pH 7.5) for 15 min. Pelleted cells were enzymatically lysed with lysis buffer prepared with buffer B (150 mM NaCl, 100 mM Tris-HCl (pH 7.8), 0.2% Triton X-100, Roche complete protease inhibitor EDTA-free) containing 1 mg ml⁻¹ lysozyme and 1 mg ml⁻¹ DNaseI and incubated on ice for 20 min followed by sonication. Cell debris were removed using centrifugation at 15,000 × g for 20 min and supernatants were centrifuged at 100,000 × g for 1 h at 4 °C to isolate envelope fraction. Sediments were solubilized with protein-denaturing buffer C (8 M urea, 100 mM Tris-HCl (pH 8.0), 1 M NaCl, 1% Triton X-100, 0.2% *N*-lauroyl sarcosine, 10 mM imidazole). His pull-down assay under denaturing condition was similar to abovementioned procedure except for using buffer C instead of buffer A in washing and eluting steps.

Bacterial two-hybrid assay

The adenylate cyclase-based two-hybrid kit (Euromedex) was employed to examine Alg8 and Alg44 interaction in a heterologous host. This experiment was designed to have a free signal peptide at N-terminal part of Alg8 and Alg44 and in a combinational manner in terms of low (pKNT25) or high (pUT18) copy number of plasmids containing the respective genes. Therefore, relevant genes were inserted at 5' end of coding region of T25 and T18 fragments of adenylate cyclase on plasmids pKNT25 and pUT18 producing plasmids pKNT25:*alg8*, pKNT25:*alg44*, pUT18:*alg8*, pUT18:*alg44*. After co-transformation of *E. coli* BTH101 competent cells with two plasmids, cells were plated on LB-X-gal-(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 20 mg/ml)-IPTG (0.5 mM) and incubated at 37°C for 48 h. Protein-protein interactions leading to functional adenylate cyclase activity were indicated by β-galactosidase activity in cell extracts of co-transformants grown in liquid culture as described (280). A level of β-galactosidase activity of at least 4-fold higher than that measured for BTH101 cells with pKNT25 and pUT18 (60-100 U mg⁻¹) or background was considered to indicate a protein-protein interaction. Each value was represented as the

mean value \pm SD from five independent experiments. Negative controls were those transformants harboring plasmids with the respective hybrid genes each together with a plain vector. The plasmids expressing leucine-zipper domains included to the kit were used as positive control.

Isolation of cytoplasmic membrane

To confirm the localization of Alg8 and Alg44 in the cytoplasmic membrane of *E. coli* BTH101, the cytoplasmic membrane fraction was isolated as described previously with some modifications (263). Overnight culture of BTH101 transformants with pBBR1MCS-5:*alg8*-6his and pBBR1MCS-5:*alg44*-6his and empty pBBR1MCS-5 in addition to *P. aeruginosa* PDO300 Δ *alg8*(pBBR1MCS-5), PDO300 Δ *alg44*(pBBR1MCS-5) (as negative controls) and PDO300 Δ *alg8*(pBBR1MCS-5:*alg8*-6his), PDO300 Δ *alg44*(pBBR1MCS5:*alg44*-6his) (as positive controls) were subcultured in 1.5 liters of LB media. Cells were harvested by centrifugation at $7,000 \times g$ and 4°C for 20 min. Tris-HCl (pH 8.3) was used for twice washing and sediments were subjected to enzymatic lysis by suspending in 20 ml of 20% sucrose in 30 mM Tris-HCl (pH 8.3), 1 mg ml^{-1} DNase I, 1 mg ml^{-1} RNase A, 1 mg ml^{-1} lysozyme and Roche complete protease inhibitor EDTA-free on ice for 20 min which was followed by sonication. Cell debris were removed and supernatants were then layered onto a sucrose density gradient containing 3 ml of 70% (wt/vol) and 3 ml of 52% (wt/vol) sucrose in Tris and centrifuged at $211,000 \times g$ for 3 h at 4°C . The buoyant layers on top of 52% sucrose step were separated for protein analysis using SDS-PAGE (8% acrylamide gels) and immunoblotting utilizing anti-6his tag antibody as will be explained in protein detection section.

Protein analysis

Protein samples were generally analysed utilizing SDS-PAGE (8% acrylamide gels). For analyzing chemically crosslinked proteins, 4-15% Mini-Protean TGX precast gradient gels (Bio-Rad) were used. Immunoblotting by using an iBlot dry-blotting system (Invitrogen) was employed to detect proteins as described previously (80) in which the antibodies anti-Alg44 (1:10,000), anti-AlgK (1:10,000), anti-AlgG (1:10,000), anti-AlgX (1:7,000), or anti-AlgE (1:5,000) in 2% skim milk and anti-Alg8 (1.5:5,000) in 2% bovine serum albumin-fraction V (Gibco/Invitrogen) were used. Treatment of membrane with commercial secondary antibody, anti-IgG anti-rabbit

antibodies labeled with horseradish peroxidase (HRP) (Abcam), followed by resolving with SuperSignal West Pico chemiluminescent substrate (Thermoscientific) were carried out and the resulting membranes were developed on X-ray film (Kodak, Rochester). For the detection of hexahistidine-tagged proteins, a HisProbe-HRP kit (Thermoscientific) was used according to the manufacturer's instruction.

Alginate purification and quantification

A total of 2 ml of bacterial overnight cultures grown in LB medium supplemented with appropriate antibiotic were harvested and washed twice with saline solution. Then, the harvested cell sediments were re-suspended in 1 ml of saline solution and then normalized to OD₆₀₀ of 3.0. A 200 μ l of cell suspension was plated onto each PIA medium (with three repetitions) containing 300 μ g ml⁻¹ of gentamicin and incubated at 37 °C for 72 h. Cells of each plate were scraped off and suspended in saline solution until all alginate materials were completely dissolved. To separate cells from alginate-containing supernatant, the suspensions were pelleted and alginates in supernatants were precipitated with equal volume of ice-cold isopropanol. Additionally, cellular sediments were freeze-dried and the final weights were determined. The alginate precipitants were freeze-dried and then re-dissolved in 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂ to a final concentration of 0.5% (wt/vol), followed by incubation with 15 μ g ml⁻¹ DNaseI and 15 μ g ml⁻¹ RNaseI at 37 °C for 6 h. Then, Pronase E was added to a final concentration of 20 μ g ml⁻¹ and incubated for further 18 h at 37 °C. Alginate solutions were dialyzed (12-14 kDa MWCO, ZelluTrans/Roth mini dialyzer, Carl Roth GmbH & Co) against 5 l of ultrapure H₂O for 48 h. Finally, alginates were precipitated with equal volume of ice-cold isopropanol and freeze-dried for uronic acid assay and biochemical analysis.

Following a modified protocol for alginate quantification or uronic acid assay (13) and using purified alginic acid from brown algae (Sigma-Aldrich) as a standard, alginate samples were dissolved in 200 μ l of ultrapure water at concentrations between 0.25 and 0.05 mg ml⁻¹. Each sample was mixed with 1.2 ml of tetraborate solution (0.0125 M disodium tetraborate in concentrated sulfuric acid) and incubated on ice for 10 min followed by incubation at 100°C for 5 min and then cooled down on ice for further 5 min. By adding 20 μ l of 3-phenylphenol reagent (0.15% of 3-phenylphenol in 0.125 M NaOH), reactions were developed within 1 min of vortexing. For each sample and dilution a negative control was assayed by using 0.125 M NaOH instead of the 3-

phenylphenol reagent. Uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

Size exclusion chromatography-multiangle laser light scattering (SEC-MALLS) analysis

The average molecular weights of the alginates produced were analysed by SEC-MALLS (Waters 2690 Alliance separations module; Waters 450 variable wavelength detector set at 280 nm; DAWN-EOS multi-angle laser light scattering detector with a laser at 690 nm (Wyatt Technology Corp., Santa Barbara, CA, USA); Waters 2410 refractive index monitor). Purified samples were dissolved in 0.1 M NaNO₃ (2 mg ml⁻¹) and allowed to hydrate fully by incubating at room temperature overnight. Immediately prior to analysis, samples were pre-heated at 80 °C for 5 min, injected (100 µl) and eluted with 0.1 M NaNO₃ (0.7 ml min⁻¹, 60 °C) from two columns (TSK-Gel G5000P_{WXL} and G4000P_{WXL}, 300×7.8 mm, Tosoh Corp.) connected in series. ASTRA software (version 6.1.1.17, Wyatt Technology Corp.) and dn/dc of 0.150 g ml⁻¹ was used for determining weight-average molecular weights (\overline{M}_w) and number-average molecular weights (\overline{M}_n) and polydispersity index (PI) via the fraction $\overline{M}_w/\overline{M}_n$. In the case of a perfectly monodisperse (homogeneous) polymer PI value equals 1.0.

¹H-nuclear magnetic resonance (NMR) spectroscopy analysis

Compositional analysis of alginate samples was done by using ¹H-NMR. The spectra were recorded at 90 °C with a JEOL 270 NMR spectrometer (6.34 T) operating at 270 MHz for proton. Samples were prepared as described by Grasdalen et al (281). The chemical shifts were expressed in ppm downfield from the signal for 3-(trimethylsilyl) propanesulfonate. The integration of the ¹H-NMR signals allowed us to determine the composition of the different deacetylated alginate samples and their acetylation degree (30, 282, 283). For alkaline deacetylation 30 ml of 1% alginates in saline solution were treated with 12 ml of 1M NaOH in 65 °C for 30 min and neutralized with 12 ml of 1M HCl. Treated samples were then dialyzed against 5 l of distilled water for 48 h and then freeze-dried.

Fourier Transform Infrared Spectrometry (FTIR) analysis

This analysis was employed to confirm the acetylation of the alginate samples and the spectra were recorded as KBr discs with a Nicolet 5700 FTIR spectrometer in the range of 400–4000 cm^{-1} . FTIR band associated with *o*-acetyl groups of the alginate were observed at 1,730 and 1,250 cm^{-1} (35, 272).

Microrheological analysis

In order to measure viscoelastic property of the alginates, the mean-square displacement (MSD) of probe particles embedded in the samples and in turn the viscoelastic moduli (G' (elastic) and G'' (viscose)) were measured (284-286). A 2.8 μl aliquot of a 2.5% (wt/vol) suspension of Polybead® polystyrene microspheres (0.5 μm) (Polysciences) was mixed with 250 μl aliquot of 0.1% alginate samples in Milli-Q water. Particle motion was recorded using an inverted microscope (Nikon Eclipse TE2000-U) on an air damped table (Photon Control) equipped with an Andor Neo CMOS camera operated at 204 fps, and a 60x 1.2 NA (Nikon, Plan Apo VC 60x WI) water immersion objective lens. Image series acquired for approximately four seconds and x-y coordinate data extracted using polyparticle tracking software (287). An in-house program was used to calculate the MSD with a program to extract the rheological information (288).

Continuous-culture flow cell biofilms and quantitative analysis

Biofilm architecture analysis was performed for those mutants producing alginates with very distinct composition and properties from each other including PDO300(pBBR1MCS-5), PDO300 Δ alg8(pBBR1MCS-5:alg8), PDO300 Δ alg44(pBBR1MCS-5:alg44), PDO300 Δ algG(pBBR1MCS-5:algG), PDO300 Δ algG(pBBR1MCS-5:algG(D324A)), PDO300 Δ algX(pBBR1MCS-5:algX), PDO300 Δ algX(pBBR1MCS-5:algX(S269A)) and PDO300 Δ algX Δ algG(pBBR1MCS-5:algX(S269A):algG(D324A)). Each mutant was grown in continuous-culture flow cells (channel dimensions of 4 mm by 40 mm by 1.5 mm) at 37 °C (264). A 500 μl suspension of cells at early-stationary-phase was injected into each channel and kept upside down for 4 hours. Then, flow was started with a mean flow of 0.3 ml min^{-1} , corresponding to a laminar flow with a Reynolds number of 5 (15, 138). The flow cells were then incubated at 37 °C for 24 h. Biofilms were stained utilizing the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) and visualized using confocal laser

scanning microscopy (Leica SP5 DM6000B). For quantitative analysis of biofilms IMARIS image analysis software (Bitplane) was employed. Biofilm architecture and appearance, biovolume (μm^3), the ratio of biovolume per unit area ($\mu\text{m}^3 \mu\text{m}^{-2}$), dead-to-live ratio, compactness and thickness of base layers were analysed (138, 265, 266).

Motility assay

Motilities including twitching, swarming and swimming were assessed by the method explained by Pang et al (267) with modification. The medium consisted of modified M9 medium (20 mM NH_4Cl ; 12 mM Na_2HPO_4 ; 8.6 mM NaCl ; 1 mM MgSO_4 ; 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and 10 mM dextrose, supplemented with 0.5% (wt/vol) Casamino acids (Difco) (15) solidified with 1% , 0.5% and 0.3% (wt/vol) Bacto agar (Difco) respectively for twitching, swarming and swimming assays. Petri dishes were kept under laminar flow for 60 min and then swarming and swimming plates were immediately inoculated with 5 μL of stationary-phase bacterial culture while for twitching stab-inoculation to the underlying petri dishes was performed using sterile toothpicks. Plates were incubated at 30 °C for 18-24 h. Swarming and swimming were assessed by measuring colony diameters and twitching zone was measured up after removing agar layer, washing free cells and staining with 1% crystal violet. All experiments were done in triplicates.

Supplemental Figures

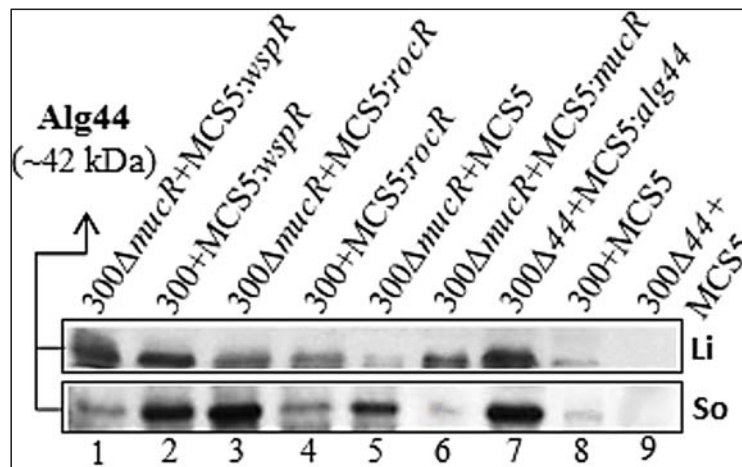


Fig. S1. Alg44 stability is independent of c-di-GMP. In envelope fractions of planktonic (Li: liquid) and biofilm (So: solid) cells and in the presence and/or absence of WspR (generating c-di-GMP) (lanes 1 and 2) and RocR (degrading c-di-GMP) (lanes 3 and 4) and MucR (alginate biosynthesis-associated c-di-GMP regulator) (lanes 5 and 6) Alg44 was detected using immunoblotting with anti-Alg44 antibody. Lanes 7-9 show negative and positive controls. 300: PDO300; MCS5:pBBR1MCS-5.

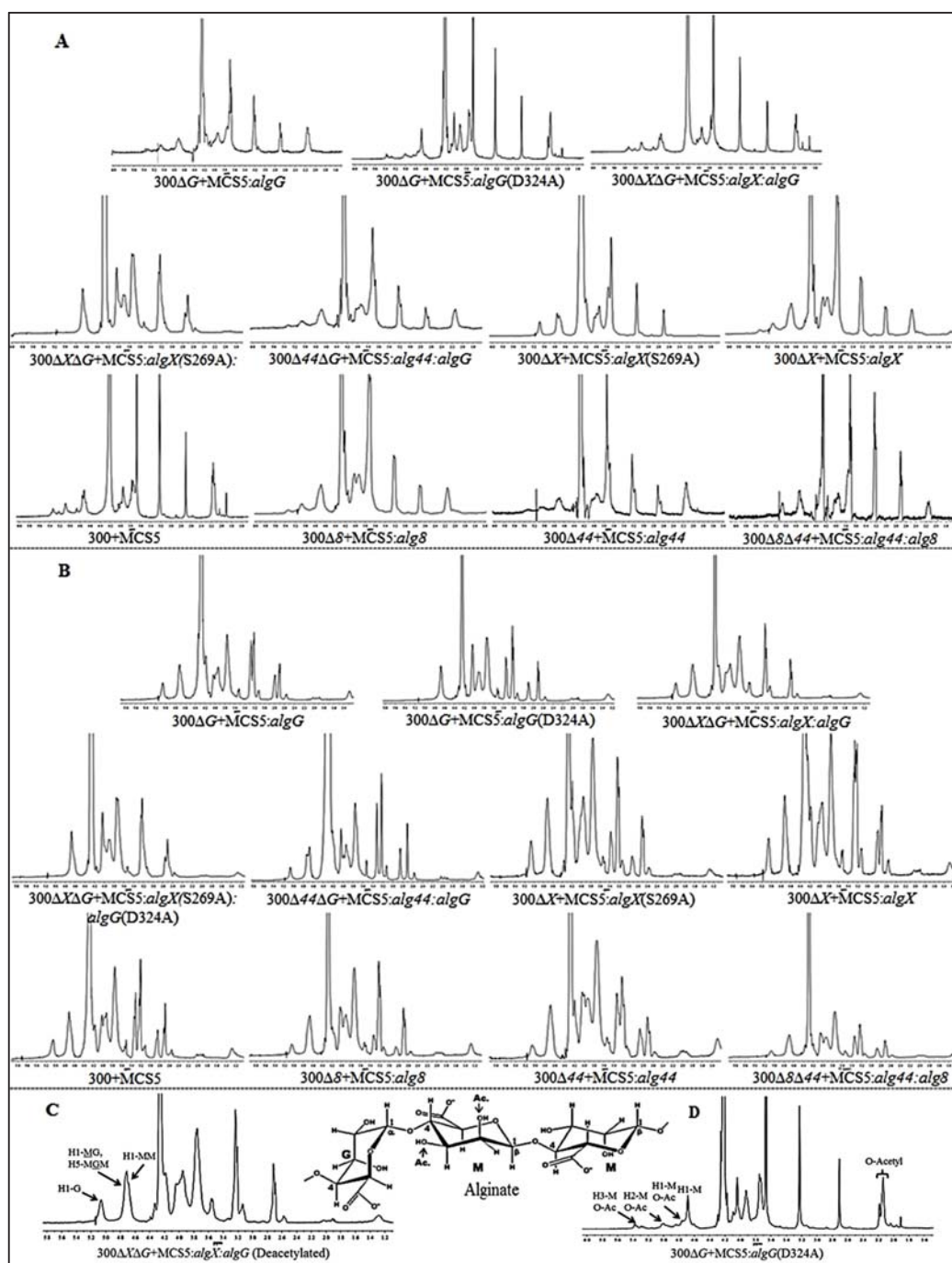


Fig. S2. $^1\text{H-NMR}$ spectra of acetylated (A) and deacetylated (B) samples. (C) Representative $^1\text{H-NMR}$ spectrum for M and G residues detection. H1-MM, H1-G and H1-MG indicate the anomeric protons of M residues followed by another M residue, G residues along the alginate chain and M residues followed by a G residue respectively. (D) Representative $^1\text{H-NMR}$ spectrum for O-acetylation indicates the methyl group of the acetyl residues along the alginate chain. H1-M indicates the anomeric proton of non-acetylated M residues. H1-M O-Ac, H2-M O-Ac and H3-M O-Ac indicates the anomeric proton, H2 and H3 of acetylated M residues.

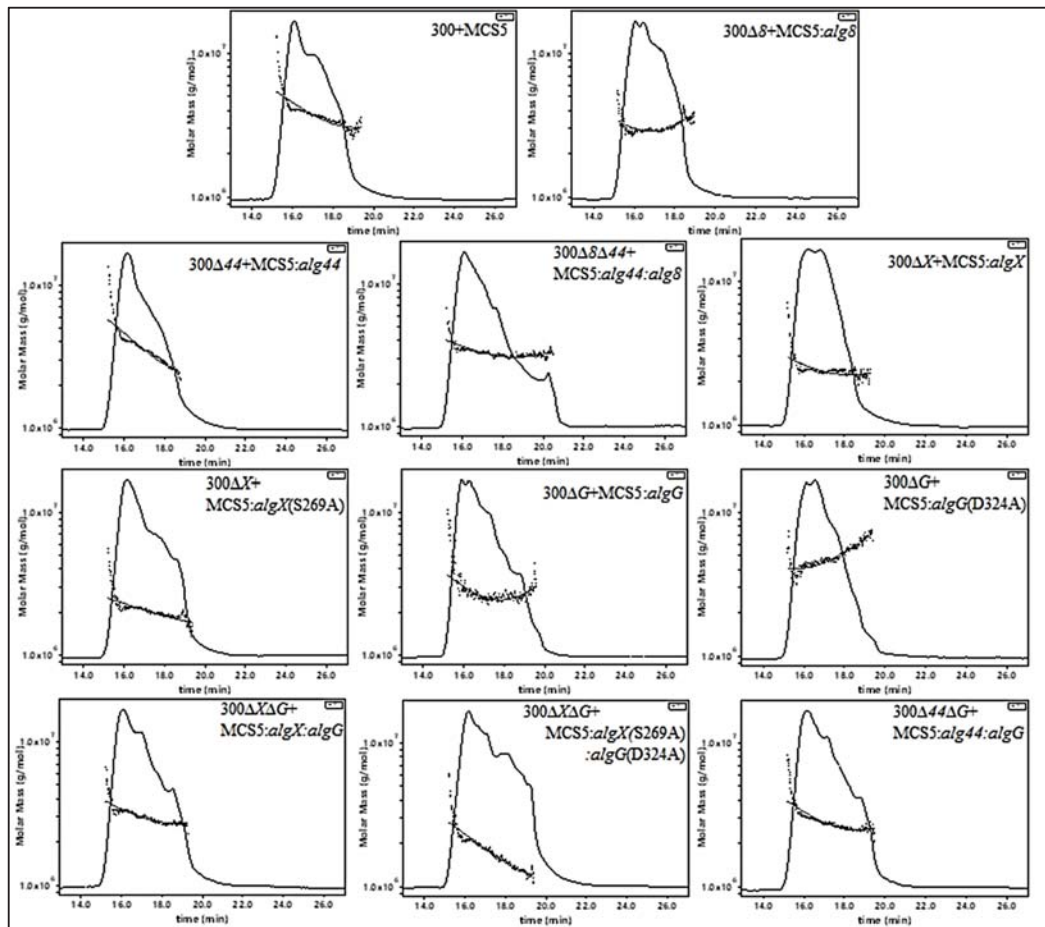


Fig. S3. Plots show molar mass of alginate samples versus time analysed using SEC-MALLS. 300:PDO300; MCS5:pBBR1MCS-5.

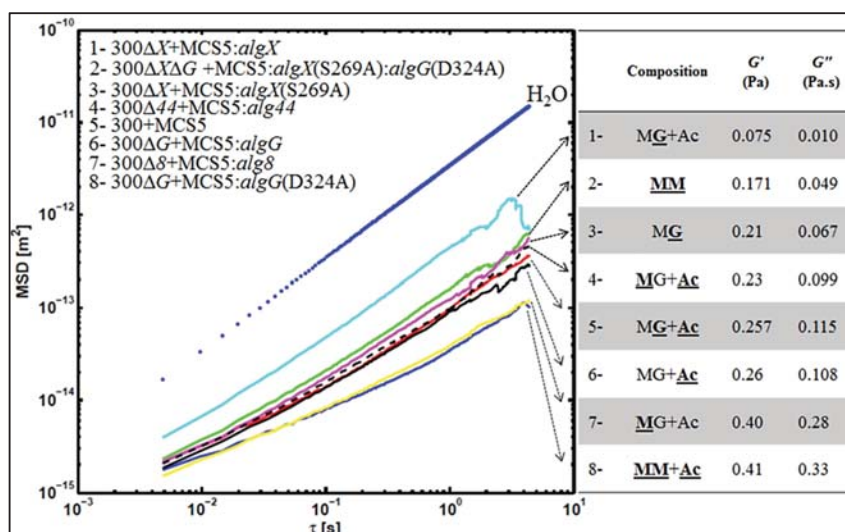


Fig. S4. Viscoelastic property of alginates was impacted by molecular weight and modifications. The plot of MSD versus time shows distribution of alginate viscoelastic properties in four distinct regions. Alginates composition and viscoelasticity moduli (G' : elastic, G'' : viscous) are presented next to the plot. Bold and underlined letters indicate the predominance of mannuronate (M)/guluronate (G) molar fraction or acetylation (Ac.) degree among all analysed alginates. 300: PDO300; MCS5: pBBR1MCS-5.

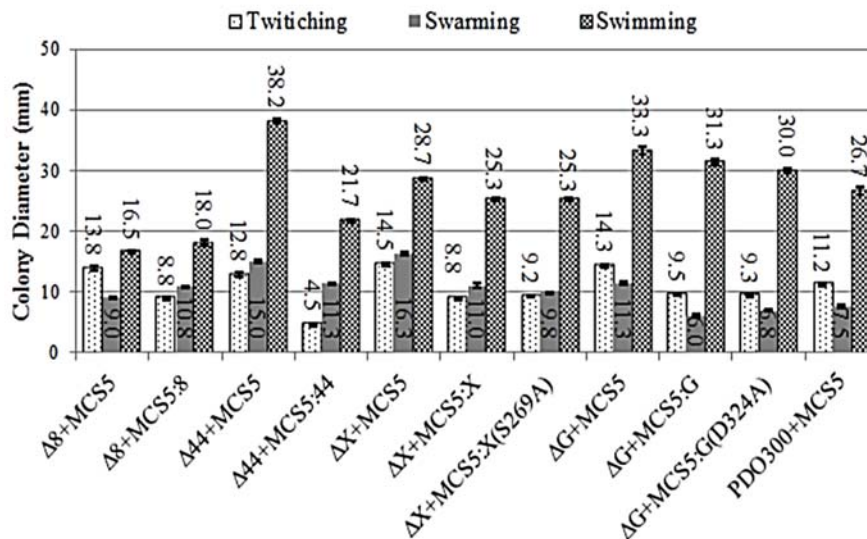


Fig. S5. Impact of alginates on motility of *P. aeruginosa*. Twitching, swarming and swimming was assessed in triplicates on 1%, 0.5% and 0.3% agar plates, respectively, and after incubation at 30 °C for 18-24 h. The mean of colony diameter values \pm standard deviation errors are graphed.

Table S1. Strains and plasmids applied in this study

Strains and plasmids	Description	Source or reference
Strains		
<i>P. aeruginosa</i>		
PDO300	<i>mucA22</i> isogenic mutant derived from PAO1, Alg ⁺	(152)
PDO300Δ <i>alg8</i>	Isogenic <i>alg8</i> deletion mutant derived from PDO300, Alg ⁻	(11)
PDO300 Δ <i>alg44</i>	Isogenic <i>alg44</i> deletion mutant derived from PDO300, Alg ⁻	(268)
PDO300 Δ <i>alg8</i> Δ <i>alg44</i>	Isogenic <i>alg8</i> and <i>alg44</i> deletions mutant derived from PDO300, Alg ⁻	This study
PDO300 Δ <i>algG</i>	Isogenic <i>algG</i> deletion mutant derived from PDO300, Alg ⁻	This study
PDO300 Δ <i>algX</i>	Isogenic <i>algX</i> deletion mutant derived from PDO300, Alg ⁻	(289)
PDO300 Δ <i>algG</i> Δ <i>algX</i>	Isogenic <i>algG</i> and <i>algX</i> deletions mutant derived from PDO300, Alg ⁻	This study
PDO300 Δ <i>algG</i> Δ <i>alg44</i>	Isogenic <i>algG</i> and <i>alg44</i> deletions mutant derived from PDO300, Alg ⁻	This study
PDO300 Δ <i>algX</i> Δ <i>alg44</i>	Isogenic <i>algX</i> and <i>alg44</i> deletions mutant derived from PDO300, Alg ⁻	This study
PDO300 Δ <i>mucR</i>	Isogenic <i>mucR</i> deletion mutant derived from PDO300, Alg ⁻	(15)
<i>E. coli</i>		
Top10	Cloning strain; F, <i>mcrA</i> , Δ(<i>mrr-hsdRMS-mcrBC</i>), φ80 <i>lacZ</i> Δ <i>M15</i> , Δ <i>lacX74</i> , <i>recA1</i> , <i>araD139</i> , Δ(<i>araleu</i>)7697 <i>galU</i> , <i>galK</i> , <i>rpsL</i> (StrR), <i>endA1</i> , <i>nupG</i>	Invitrogen
XL1 Blue	Cloning strain; <i>ecA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>hsdR17</i> , (<i>r_K⁻</i> , <i>m_K⁺</i>), <i>supE44</i> , <i>relA1</i> , Δ(<i>lac-proAB</i>) [<i>F⁺</i> . <i>proAB</i> , <i>lacI^s</i> , <i>lacZ</i> Δ <i>M15</i> , Tn10(<i>tet^r</i>)]	(290)
S17-1	Donor strain in transconjugation; <i>thi-1</i> , <i>proA</i> , <i>hsdR17</i> (<i>r_K⁻</i> <i>m_K⁺</i>), <i>recA1</i> ; <i>tra</i> gene of plasmid RP4 integrated in chromosome	(244)
SM10	Donor strain for pFLP2 plasmid; <i>thi-1</i> , <i>thr-1</i> , <i>leuB6</i> , <i>supE44</i> , <i>tonA21</i> , <i>lacY1</i> , <i>recA</i> ::RP4-2-Tc::Mu Km ^r	(244)
BTH101	Protein-protein interaction strain; F, <i>cya-99</i> , <i>araD139</i> , <i>galE15</i> , <i>galK16</i> , <i>rpsL1</i> (Sp ^R), <i>hsdR2</i> , <i>mcrA1</i> , <i>mcrB1</i>	Euromedex
Plasmids		
pBBR1MCS-5	Gm ^r ; broad-host-range vector; P _{lac}	(246)
pBBR1MCS-5: <i>alg8</i>	<i>HindIII-PstI</i> fragment comprising <i>alg8</i> inserted into vector pBBR1MCS-5	(11)
pBBR1MCS-5: <i>alg8</i> -6his	Translational Alg8-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	(13)
pBBR1MCS-5: <i>alg44</i>	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> inserted into vector pBBR1MCS-5	(268)
pBBR1MCS-5: <i>alg44</i> (R21D)-6his	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding translational Alg44-hexahistidine tag fusion with site-directed mutagenesis R21A inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> (R17A)	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding site-directed mutagenesis R17A inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> (R21A)	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding site-directed mutagenesis R21A inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> (R17A, R21A)	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding site-directed mutagenesis R17A and R21A inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> (R17A, R21A): <i>rocR</i>	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding site-directed mutagenesis R17A and R21A and <i>XbaI-SacI</i> fragment encoding RocR (PA3947) inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> (Δ40-74aa _{PII2})-6his	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding translational Alg44 with N-terminal oligonucleotide deletion (residues 40-74) inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> (Δ364-389aa)-6his	<i>HindIII-BamHI</i> fragment comprising <i>alg44</i> encoding translational Alg44 with C-terminal oligonucleotide deletion (residues 364-389) inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>alg44</i> -6his	Translational Alg44-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	This study

pBBR1MCS-5: <i>algG</i>	<i>Bam</i> HI- <i>Xba</i> I fragment comprising <i>algG</i> inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>algG</i> (D324A)	<i>Bam</i> HI- <i>Xba</i> I fragment comprising <i>algG</i> encoding site-directed mutagenesis D324A inserted into vector pBBR1MCS-5; mannuronic acid(M)-epimerase activity	This study
pBBR1MCS-5: <i>algG</i> -6his	Translational AlgG-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>algX</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>algX</i> inserted into vector pBBR1MCS-5	
pBBR1MCS-5: <i>algX</i> -6his	Translational AlgX-hexahistidine tag fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>algX</i> (S269A)	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>algX</i> encoding site-directed mutagenesis S269A inserted into vector pBBR1MCS-5; Acetyltransferase activity	This study
pBBR1MCS-5: <i>algX:algG</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>algX</i> and <i>Bam</i> HI- <i>Xba</i> I fragment comprising <i>algG</i> inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>algX</i> (S269A): <i>algG</i> (D324A)	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>algX</i> encoding site-directed mutagenesis S269A and <i>Bam</i> HI- <i>Xba</i> I fragment comprising <i>algG</i> encoding site-directed mutagenesis D324A inserted into vector pBBR1MCS-5; M-epimerase activity, Acetyltransferase activity	This study
pBBR1MCS-5: <i>alg44:algG</i>	<i>Cla</i> I- <i>Bam</i> HI fragment comprising <i>alg44</i> and <i>Bam</i> HI- <i>Xba</i> I fragment comprising <i>algG</i> inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5: <i>algX:alg44</i>	<i>Cla</i> I- <i>Hind</i> III fragment comprising <i>algX</i> and <i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>alg44</i> inserted into vector pBBR1MCS-5	(80)
pBBR1MCS-5: <i>mucR</i>	<i>Kpn</i> I- <i>Cla</i> I fragment comprising <i>mucR</i> inserted into vector pBBR1MCS-5	(15)
pBBR1MCS-5: <i>wspR</i>	<i>Xba</i> I- <i>Sac</i> I fragment comprising <i>wspR</i> inserted into vector pBBR1MCS-5	(15)
pBBR1MCS-5: <i>rocR</i>	<i>Xba</i> I- <i>Sac</i> I fragment comprising <i>rocR</i> inserted into vector pBBR1MCS-5	(15)
pEX100T	Ap ^r Cb ^r , gene replacement vector containing <i>sacB</i> gene for counterselection	(242)
pEX100T:Δ <i>alg8</i> Gm	Ap ^r Cb ^r , Gm ^r , vector pEX100T with <i>Sma</i> I-inserted <i>alg8</i> deletion construct	(11)
pEX100T:Δ <i>alg44</i> Gm	Ap ^r Cb ^r , Gm ^r , vector pEX100T with <i>Sma</i> I-inserted <i>alg44</i> deletion construct	(268)
pEX100T:Δ <i>algG</i> Gm	Ap ^r Cb ^r , Gm ^r , vector pEX100T with <i>Sma</i> I-inserted <i>algG</i> deletion construct	This study
pFLP2	Ap ^r Cb ^r ; broad-host-range vector encoding Flp recombinase	(242)
pGEM-T Easy	Cloning vector; Amp ^r ; T-overhang cloning	Promega
pGEM-T Easy: <i>alg44</i> -6his	A-tailed fragment encoding C-terminally hexahistidine-tagged Alg44 inserted into pGEM-T Easy	This study
pGEM-T Easy: <i>alg44</i> (Δ364-389aa)	A-tailed fragment encoding C-terminally hexahistidine-tagged Alg44 inserted into pGEM-T Easy	This study
pGEM-T Easy: <i>alg8</i> -6his	A-tailed fragment encoding C-terminally hexahistidine-tagged Alg8 inserted into pGEM-T Easy used for sequencing	This study
pGEM-T Easy: <i>algX</i> -6his	A-tailed fragment encoding C-terminally hexahistidine-tagged AlgX inserted into pGEM-T Easy used for sequencing	This study
pGEM-T Easy: <i>algG</i> -6his	A-tailed fragment encoding C-terminally hexahistidine-tagged AlgG inserted into pGEM-T Easy used for sequencing	This study
Mini-CTX-lacZ	Chromosomal integration vector at the CTX phage <i>att</i> site on the <i>P. aeruginosa</i> chromosome, Tc ^r	(291)
Mini-CTX: <i>Palg44</i> -6his	<i>Pst</i> I- <i>Hind</i> III fragment encoding alginate operon promoter (<i>PalgD</i>) and <i>Hind</i> III- <i>Bam</i> HI fragment encoding C-terminally hexahistidine-tagged Alg44 inserted into vector Mini-CTX-lacZ	This study
Mini-CTX: <i>Palg8</i> -6his	<i>Pst</i> I- <i>Hind</i> III fragment encoding alginate operon promoter (<i>PalgD</i>) and <i>Hind</i> III- <i>Bam</i> HI fragment encoding C-terminally hexahistidine-tagged Alg8 inserted into vector Mini-CTX-lacZ	This study
Mini-CTX: <i>PalgG</i> -6his	<i>Pst</i> I- <i>Hind</i> III fragment encoding alginate operon promoter (<i>PalgD</i>) and <i>Hind</i> III- <i>Bam</i> HI fragment encoding C-terminally hexahistidine-tagged AlgG inserted into vector Mini-CTX-lacZ	This study
Mini-CTX: <i>PalgX</i> -6his	<i>Pst</i> I- <i>Hind</i> III fragment encoding alginate operon promoter (<i>PalgD</i>) and <i>Hind</i> III- <i>Bam</i> HI fragment encoding C-terminally hexahistidine-tagged AlgX	This study

pKNT25	inserted into vector Mini-CTX-lacZ 3.4-kb plasmid allowing C-terminal protein fusion to T25 fragment; Kan ^r	Euromedex
pKNT25: <i>alg8</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>alg8</i> (Δ stop) cloned into pKNT25 upstream of T25 fragment	This study
pKNT25: <i>alg44</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>alg44</i> (Δ stop) cloned into pKNT25 upstream of T25 fragment	This study
pKT25-zip	Derivative of pKNT25, having an leucine zipper motif of GCN4 fused in frame to the C-terminal end of CyaA _{T25} ; Kan ^r	Euromedex
pUT18	3.0-kb plasmid allowing C-terminal protein fusion to T18 fragment; Amp ^r	Euromedex
pUT18: <i>alg8</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>alg8</i> (Δ stop) cloned into pUT18 upstream of T18 fragment	This study
pUT18: <i>alg44</i>	<i>Hind</i> III- <i>Bam</i> HI fragment comprising <i>alg44</i> (Δ stop) cloned into pUT18 upstream of T18 fragment	This study
pUT18C-zip	Derivative of pUT18, having an leucine zipper motif of GCN4 fused in frame to the C-terminal end of CyaA _{T18} ; Amp ^r	Euromedex

Chapter VI

The role of Alg44 in alginate synthesis and modification

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Abstract

The exopolysaccharide, alginate, is a virulence factor produced by the ubiquitous opportunistic human pathogen *Pseudomonas aeruginosa*. It is synthesized by a proposed envelope-spanning multiprotein complex. Alg44 is an inner membrane protein and subunit of the multiprotein complex directly involved in alginate polymerisation mediating activation of polymerisation through a cytoplasmic PilZ domain upon binding of c-di-GMP. The role of the periplasmic domain of this protein is still unknown. Here, bioinformatic analysis and site-specific mutagenesis showed that highly conserved and clustered residues QMK (residues 258-260) and TSPCDC (residues 264-269) are essential for alginate production. Alanine substitution of P266, C267 and C269 abolished alginate production by destabilising Alg44. Replacement of the other amino acid residues in these two motifs caused alginate production reduction ranging from 71% to 99.6%. These mutations also caused significant reduction of acetylation, by up to 30%, while epimerisation was increased by up to 114%. D268A and M259A variants showed the lowest level of acetylation and the highest level of epimerisation activity. Alg44 was successfully purified from *P. aeruginosa* PDO300 Δ alg44 containing chromosomally integrated gene encoding His-tagged Alg44. Purified His-tagged Alg44 formed a stable dimer. Overall, this study sheds light on the structure-function relationships of Alg44 periplasmic domain and demonstrated that Alg44 is not only directly involved in alginate polymerization but that it is also impacting on alginate modification.

Introduction

Pseudomonas aeruginosa is the leading cause of chronic bronchopulmonary infection in cystic fibrosis patients and many nosocomial infections. The secretion of extracellular polymeric substances leads to the formation of a developed biofilm, protecting embedded bacteria against the immune response and antibiotic treatments. One of these extracellular biofilm matrix components is alginate, an anionic exopolysaccharide composed of variable proportions of 1,4-linked β -D-mannuronic acid (M) and its C-5 epimer α -L-guluronic acid (G). Alginate is known as a virulence factor involved in development of characteristic biofilms of *P. aeruginosa* (95, 292-294). The extracellular alginate layer in mucoid strains reduces diffusion of antibiotics, scavenges free radicals released by triggered mechanisms in the immune system and interferes with polymorphonuclear leukocyte (PMN)-mediated killing (3-5). The *P. aeruginosa* alginate is synthesized by an envelope-spanning multiprotein complex consisting of Alg8, Alg44, AlgG, AlgX, AlgK and AlgE including possible involvement of AlgI-AlgJ-AlgF (required for acetylation) and AlgL (80, 279, 295). These proteins are responsible for polymerisation, translocation, modification (acetylation and epimerisation) and secretion of alginate (80, 279, 295). Two cytoplasmic-anchored proteins, Alg8 and Alg44, are associated into a catalytic unit for polymerisation process. Polymerisation is activated by binding of a dimeric form of bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) to Alg44 PilZ domain in cytoplasm (295, 296). During translocation of nascent polymannuronic (poly-M) chain across the periplasm, some M residues are epimerised to G residues by AlgG to form poly-MG chains (16, 21). Additionally, AlgX and AlgJ independently perform enzymatic O-acetylation of alginate (22, 23). Recently, experimental evidence was provided that epimerisation and O-acetylation are linked and AlgX and AlgG play auxiliary roles for each other's functions (295). The correlation of alginate molar mass and production rate with these modifications has also been demonstrated (295). Epimerization was found to be inversely correlated with the molecular weight, but had a positive impact on production rate. In contrast, high levels of acetylation were coinciding with higher molecular weight of alginates. AlgK and AlgE were found to interact with each other and constitute the secretion function of the complex (20, 57, 80). AlgL is responsible for degrading misguided alginate into the periplasmic space (25, 67, 297, 298).

Recently, increased copy numbers of Alg44 was found to boost acetylation of alginate through a hitherto unknown mechanism (295). Alg44 is localized to the cytoplasmic membrane interaction with the glycosyltransferase Alg8. Its cytoplasmic PilZ domain mediates c-di-GMP dependent activation of alginate polymerization. In addition, it contains a periplasmic domain whose function has not been analysed yet. In this study, we investigated the proposed periplasmic domain of Alg44 using bioinformatic analysis and site-specific mutagenesis to study the role of some highly conserved and clustered residues with respect to alginate production, polymerisation, composition/modification, protein stability and localization. Furthermore, Alg44 was purified and its quaternary structure analysed.

Material and Methods

Bacterial strains, plasmids, and growth condition

P. aeruginosa and *Escherichia coli* strains were cultivated in Luria Broth medium supplemented by appropriate antibiotics and were grown at 37°C. Alginate production and phenotypic assessments were performed using Difco™ Pseudomonas Isolation Agar (PIA) medium supplemented by appropriate antibiotics. *E. coli* strains including C41(DE3), C43(DE3), BL21(DE3), Origami™ (DE3), ClearColi® and Rosetta™ strains applied for heterologous production of protein were purchased from Lucigen or Novagen. Large-scale cell culture preparations were performed in Luria Broth medium. All chemicals were purchased from Sigma-Aldrich and Merck KGaA unless otherwise mentioned. All applied enzymes used for cloning were manufactured by Roche, New England Biolabs GmbH or Invitrogen. Alginate powder (from brown algae) was purchased from Sigma-Aldrich.

Construction of isogenic mutant with knockout of *alg44* gene

The *P. aeruginosa* PDO300 was used to generate isogenic single-gene knockout mutant in *alg44* gene as described previously (221). Briefly, this was performed through two events of homologous recombination using suicide plasmid pEX100T. This plasmid contained knockout genes which were disrupted by the *aacCI* gene (encoding gentamicin acetyltransferase) flanked by two *FRT* sites. Removing the *FTR-aacCI-FRT* cassette was performed by transfer the flippase recombinase encoding vector pFLP2 (242) into presumable knockout mutants resulting in *P. aeruginosa* PDO300 Δ *alg44*

mutant which were confirmed using antibiotic-sensitivity screening and PCR with *alg44* up/down primers (59, 221, 245).

***In trans* and *in cis* complementation of knockout mutant in *alg44* gene and *E. coli* strains transformation**

The genes encoding Alg44 or Alg44-6His were transferred into generated mutants using pBBR1MCS-5 (246) plasmid and also incorporated into the genome using mini-CTX-lacZ plasmid (248) as described previously in detail (80, 221, 295). The vector pETDuet-1 (Novagen) was applied to construct pETDuet-1:*alg44*-12His for heterologous production of Alg44-12His. Relevant encoding gene was optimized based on *E. coli* codon usage and synthesized by GenScript.

Site-specific mutations of *alg44* gene

Each residue of the clusters QMK (residues 258-260) and TSPCDC (residues 264-269) were mutated to alanine using DNA synthesis (GenScript) and ligated into corresponding region on *alg44* using *SapI* and *BamHI* sites. The resultant plasmids were pBBR1MCS-5:*alg44*(Q258A)-6His, pBBR1MCS-5:*alg44*(M259A)-6His, pBBR1MCS-5:*alg44*(K260A)-6His, pBBR1MCS-5:*alg44*(T264A)-6His, pBBR1MCS-5:*alg44*(S265A)-6His, pBBR1MCS-5:*alg44*(P266A)-6His, pBBR1MCS-5:*alg44*(C267A)-6His, pBBR1MCS-5:*alg44*(D268A)-6His, pBBR1MCS-5:*alg44*(C269A)-6His, pBBR1MCS-5:*alg44*(C267A/C269A)-6His.

Alginate purification and quantification and free uronic acid assay

Two ml of bacterial overnight culture grown in LB medium supplemented with the appropriate antibiotic were sedimented and cells washed twice with saline solution. Cells were suspended in 1 ml of saline solution and 200 μ l of cell suspension was plated onto PIA medium (in triplicates) containing 300 μ g ml⁻¹ of gentamicin and then incubated at 37 °C for 72 h. Grown cells were scraped off from agar plates and suspended in saline solution until homogenous suspension was formed. Then suspensions were pelleted and supernatants containing alginate were precipitated with equal volume of ice-cold isopropanol. The alginate precipitants were freeze-dried and then re-dissolved in 50 mM Tris-HCl pH 7.4, 10 mM MgCl₂ to a final concentration of 0.5% (wt/vol), followed by incubation with 15 μ g ml⁻¹ DNaseI and 15 μ g m l⁻¹ RNaseI at 37 °C for 6 h. Then, Pronase E was added to a final concentration of 20 μ g ml⁻¹ and

incubated for further 18 h at 37 °C. Alginate solutions were dialyzed (12-14 kDa MWCO, ZelluTrans/Roth mini dialyzer, Carl Roth GmbH & Co) against 5 l of ultrapure H₂O for 48 h. Finally, alginates were precipitated with equal volume of ice-cold isopropanol and freeze-dried for uronic acid assay and biochemical analysis which was previously described in detail (36, 295, 299).

Assessment of free uronic acids was performed using 2 ml of overnight liquid culture. Cells were pelleted and supernatants were filtered through a Vivaspin-500 (GE Healthcare) filter device with a molecular mass cutoff of 10 kDa. The uronic acids in the flowthrough which consist of free uronic acids and short alginate degradation products and total sample were measured according to previously described procedure (300).

The analysis of composition of the alginates

Compositional analysis of alginate samples harvested from solid cultures was done by using ¹H-NMR for two different sets of original and deacetylated samples. Preparation of samples was done according to previously described method (30, 282, 295). A ten-milligram partially hydrolyzed samples were dissolved in 2 mL of D₂O (99.96%) with shaking for 2 h and lyophilized. This deuteration step was repeated twice. The deuterium-exchanged alginates (6 mg) were dissolved in 0.7 mL of D₂O. ¹H-NMR spectra were recorded at 85°C using 500 MHz Bruker Avance spectrometers. Spectra were interpreted as previously described by Grasdalen et al. (281, 295).

Protein production, cell disruption and preparation of membrane fraction

The strains of *E. coli* with pETDuet-1:*alg44*-12His were grown overnight. The main cultures of LB media supplemented with 70 µg/ml of ampicillin were inoculated with 1% (vol/vol) of overnight cultures and incubated at 37°C until OD₆₀₀ reached 0.5-0.6. The cultures were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and subsequently grown at 25°C. The *cis*-complemented PDO300Δ*alg44* strain containing ectopically inserted *alg44*-6His was grown at 37°C overnight for mass production of cells.

Cells were harvested and washed twice with sodium phosphate buffer. After disruption using sonication or microfluidizer, unbroken cells and cellular debris were removed using centrifugation at 8,000 × g for 45 min at 4°C. Supernatants were subjected to ultracentrifugation at 100,000 × g for 90 min at 4°C to isolate the envelope fraction.

Pelleted membrane fractions were dissolved at 4°C for 2 h with buffer A (800 mM NaCl, 50 mM NaH₂PO₄, 10% glycerol, 5 mM EDTA, 1.5% Triton X-100, 5 mM imidazole and Roche complete protease inhibitor EDTA-free, pH 7.7). Insoluble part was pelleted again at 100,000 × *g* for 30 min at 4°C. Supernatants were subjected to protein purification and analysis.

Protein purification and analysis

His-Spin protein miniprep kit (Zymo Research) was employed for preliminary purification assessment. Roche cOmplete His-tag purification resin (EDTA-compatible) was employed for large-scale purification of His-tagged protein. Solubilized membrane fraction was mixed with resin pre-equilibrated in buffer A (a total of 5 mL) and incubated at 4°C for 6 h with gentle shaking. The mixture was packed into columns for gravity flow and washed with 10 column volumes (cv) of buffer A. Elution was performed with 5 cv of buffer B (similar to buffer A with 500 mM imidazole, pH 7.7). A 250 µl of eluent was loaded on to a Superdex 200 Increase 10/300 GL column. Two column volumes of buffer C (300 mM NaCl, 50 mM NaH₂PO₄, 10% glycerol, 5 mM EDTA, 0.02% Triton X-100, pH 7.8) was passed through the column at 0.6 ml/min and the absorbance at 280 nm was monitored. Fractions were collected in 0.5 ml steps and subsequently assessed by SDS-PAGE (8% acrylamide gels) either with staining with Coomassie Brilliant Blue solution or immunoblotting. Immunoblotting was performed using an iBlot dry-blotting system (Invitrogen) as described previously (80). For the detection of His-tagged protein, a HisProbe-HRP kit (Thermoscientific) was used according to the manufacturer's instruction followed by resolving with SuperSignal West Pico chemiluminescent substrate (Thermoscientific) and developing on X-ray film (Kodak, Rochester). Protein sequencing was performed by The Centre for Protein Research (CPR) of Otago University.

Results

Periplasmic domain of Alg44 is crucial for alginate polymerisation.

Previously, it was demonstrated that two cytoplasmic membrane-anchored proteins, Alg8 and Alg44, constitute an active alginate polymerase subunit (295). Previous studies suggested Alg44 consists of three distinct domains, a cytoplasmic PilZ domain, a transmembrane domain and a periplasmic domain (58). Bioinformatic analysis of Alg44 suggested that the periplasmic domain of Alg44 resembles the structure of

membrane fusion proteins (MFPs) such as MexA (58, 301). Alg44 plays a crucial role in alginate production and activation through interaction with other periplasmic subunits of alginate biosynthesis/secretion complex and through binding to c-di-GMP (295). Previously, we demonstrated that site-specific mutagenesis and cytoplasmic truncation of Alg44 did not disrupt Alg44 localization, stability and protein-protein interactions (295). These studies focused on the cytoplasmic PilZ domain of Alg44, while the role of periplasmic part of this protein was not investigated.

Bioinformatic analysis using the Universal Protein Resource (UniProt) server showed highly conserved clustered residues Q258MKGTLTSPCDC269 in the periplasmic domain of Alg44 among alginate-producing *Pseudomonas* and *Azotobacter* species (Fig. 1). Based on the analysis using the Phyre2 Protein Fold Recognition Server and NCBI's conserved domain database, the folding of this region may resemble biotinyl-lipoyl-domains of biotin/lipoyl attachment proteins (302, 303). Another highly conserved feature was the existence of two adjacent cysteine residues (CXC motif) which is required for stability or functionality of various proteins within the periplasm of bacteria or in the mitochondrial intermembrane space (304-306).

Using site-specific mutagenesis approaches, the impact of alanine substitutions of these residues on alginate production was assessed. Site-specific mutagenesis of these residues either abolished or significantly reduced alginate production compared to wild-type and PDO300 Δ alg44(pBBR1MCS5:alg44-6His) (Fig. 2). Except for the variant D268A which produced small amounts of alginate, alanine substitution of each residue in the PCDC (residues 266-269) cluster abolished alginate production. Additionally, alanine substitution of M259 resulted in significant decrease in alginate production (Fig. 2).

Fig. 1. Clustered periplasmic residues of Alg44 which are highly conserved among alginate-producing species. Uniprot analysis of periplasmic part of Alg44 showed the alignment of highly conserved regions among different alginate-producing species. PA (*P. aeruginosa*), AZ (*Azotobacter vinelandii*), PF (*P. fluorescens*), PP (*P. putida*) and PS (*P. syringae*).

	Accession	
PA	Q9HY69	GHOMKGTLTSPCDCR
AZ	M9YGH0	TRQMKGTLTSPCDCK
PF	I4K0K8	GKOMTGTLTSPCDCI
PP	N9U933	GKQLSGTLTSPCDCV
PS	K2U289	GKOMSGTMTSPCDCV
		:*:*:**:*****

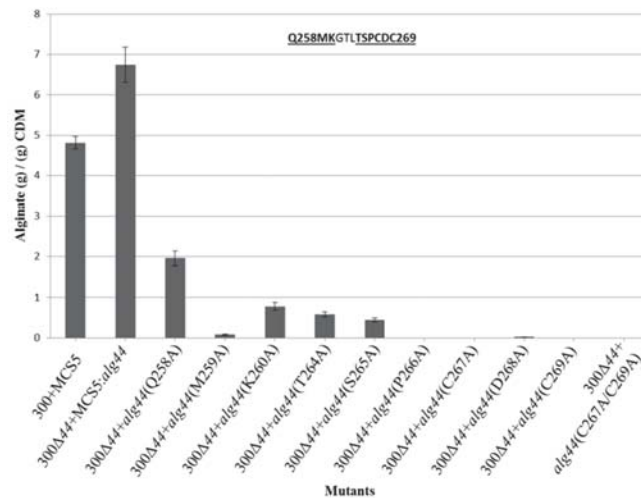


Fig. 2. Point-mutation of highly conserved periplasmic residues reduced or abolished alginate production. Highly conserved periplasmic amino acids were chosen to be replaced by alanine using site-specific mutagenesis. Alginate quantification showed P266A, C267A and C269A completely abolished alginate production and for other residues it was significantly reduced. Data are means and standard deviations of triplicates. CDM: cell dry mass; 300:PDO300; ND: Not detectable; MCS5: pBBR1MCS-5.

To assess whether the loss or reduction of alginate production and mucoid phenotype were caused by the loss of alginate polymerisation or was due to alginate degradation in the periplasm, uronic acid assay was performed using liquid medium. In this assay, high and low molecular weight alginates were separated using filters (10 kDa cutoff). Flow through contains low molecular weight alginates or free uronic acids that are presumably degraded by AlgL activity mainly due to destabilization of proposed protein scaffold in the periplasm. While, undetectable or low amount of high and low molecular weight alginates indicates alginate polymerization failed.

Reduction of total uronic acid in liquid culture was observed for all variants of Alg44 (Fig. 3). For P265A, C266A and C269A, free uronic acids of total fractions (i.e. high and low molecular mass alginate) and the relevant flow-through from filter (10 kDa cutoff) was equal, both significantly lower than the values obtained for wild-type and PDO300Δ*alg44*(pBBR1MCS5:*alg44*-6His) (Fig. 3), indicating these amino acids might be involved in the alginate polymerization function of Alg44.

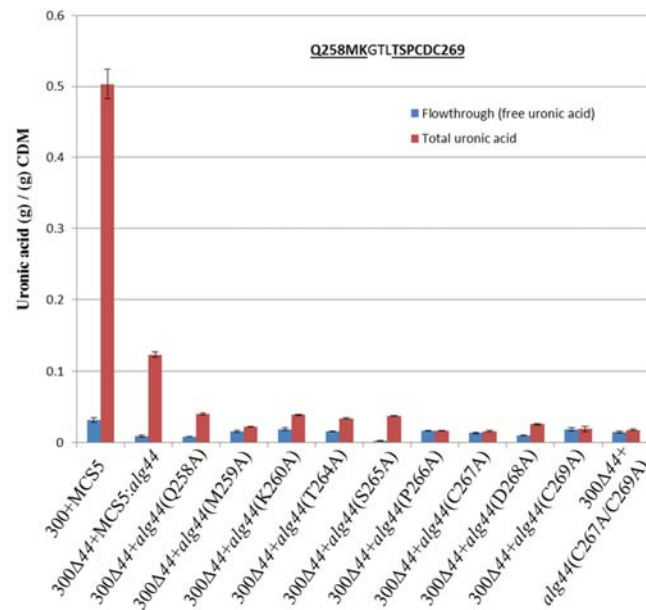


Fig. 3. Alginate polymerisation was impaired by site-specific mutagenesis of highly conserved periplasmic amino acid residues of Alg44. This figure shows the production of free uronic acids in liquid culture mediated by variants of Alg44. Data are means and standard deviations of triplicates. “Flowthrough” indicates the fraction that passed through the filter (10 KDa cutoff) and contained low molecular weight alginates or free uronic acids. “Total” indicates whole sample (without filtration) and a mix of high and low molecular weight alginates. CDM: cell dry mass; 300:PDO300; ND: Not detectable; MCS5: pBBR1MCS-5.

Stability and localization of Alg44 is impacted by periplasmic amino acid residues

In order to assess whether impairing or abolishing alginate production in each strain harboring Alg44 variants was due to a loss in stability or mis-localization of Alg44, envelope fractions of each strains were analysed using immunoblotting and anti-His-tag antibodies. Interestingly, no Alg44 variant corresponding to alanine substitution of M259, P266, C267, D268 and C269, respectively, could be detected (Fig. 4). This experiment provided the first experimental evidence showing Alg44 stability is dependent on some highly conserved residues of periplasmic domain.

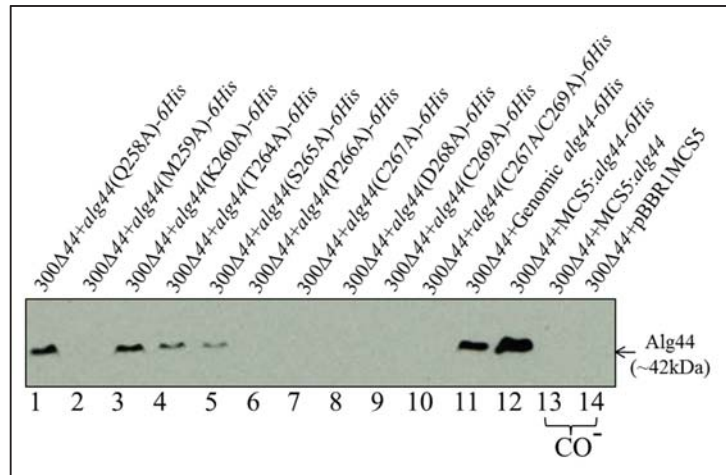


Fig. 4. Stability of Alg44 variants in envelope fraction. Immunoblot of the envelope fraction developed using anti-His-tag antibody showed that P266A, C267A and C269A completely disrupted Alg44 localization in the envelope fraction (lanes 6-7 and 9-10). Bands related to M259A and D268A were developed very faintly (lanes 2 and 8). The intensity of other bands was consistent to the amount of alginate produced by complemented mutants (lanes 1, 3-5 and 12). Lanes 13 and 14 represent negative controls. 300: PDO300; MCS5: pBBR1MCS-5; Co⁻: negative control.

Disulfide bond mediates Alg44 interaction only in native stoichiometry

As mentioned above, the two cysteine residues (residues 267 and 269) of Alg44 located in the highly conserved periplasmic domain are crucial for protein stability. It was further investigated whether these residues formed disulfide bond required for stability of protein, or were involved in inter- and intramolecular interactions. Immunoblotting analysis of DTT-treated and untreated membrane fractions of PDO300 Δ alg44 complemented with *alg44*-6His either *in cis* or *in trans* expression showed a higher molecular weight band (~70 kDa) when Alg44 was encoded *in cis* and in the absence of DTT, while it was missing when treated with DTT i.e. under reducing conditions (Fig. 5). This difference equals to ~25 kDa which is close to the molecular weight of AlgF, a subunit involved in acetylation of alginate which has two cysteine residues in its structure. This 70 kDa band was not detected when Alg44 was encoded *in trans*. The lack of Alg44 in samples corresponding to alanine substitution of cysteine residues re-confirmed their role in stability of Alg44 (Fig. 5).

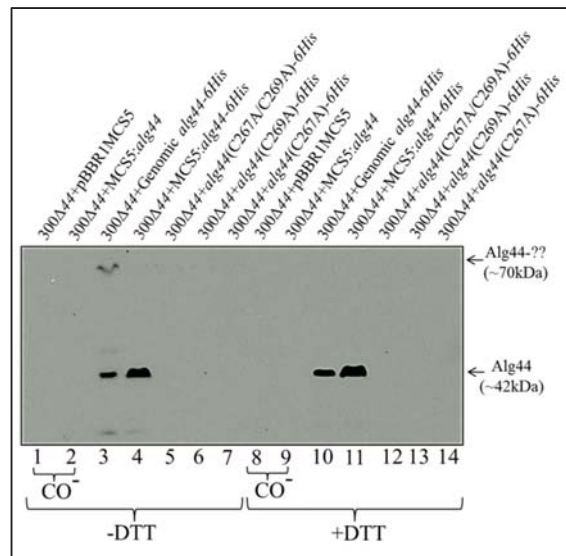


Fig. 5. Disulfide bond may play in Alg44 interaction with other subunits when it is in native stoichiometry. Immunoblot of the envelope fraction developed using anti-His-tag antibody showed that a band with higher molecular mass (~70 kDa) was detected without DTT treatment (lane 3) while it was missing after adding DTT (lane 10) or when protein was produced from plasmid (lanes 4 and 11). Immunoblot confirmed Alg44 was missing when cysteine residues were replaced with alanine (lanes 5-7 and 12-14). Lanes 1-2 and 8-9 represent negative controls. 300: PDO300; MCS5: pBBR1MCS-5; Co⁻: negative controls.

Alg44 regulates modification of alginate through its periplasmic domain

Although AlgX and AlgJ have been understood to be independent acetyltransferases (22) and other subunits including AlgI and AlgF mediate acetylation of alginate (23, 24, 272), the functional and structural relationship of these subunits and the pathway of supplying possible precursor(s) are unknown. Previously, we demonstrated that when Alg44 was presented in additional copy, acetylation degree of alginate was enhanced, while the molar fraction of MM-block in the polymer increased (295). Alg44 was also shown to interact with AlgX which is an acetyltransferase of the alginate polymerisation/secretion protein complex (295).

Here, bioinformatic analysis using NCBI and the Phyre2 Protein Fold Recognition Server predicted that the conserved clustered residues of Alg44 were part of a domain whose structure was modelled on biotinyl-lipoyl-domains which are present in a family of proteins that have carboxylase/decarboxylase and acyltransferase activity (302). The function of these domains is to transport functional groups such as CO₂ and acyl between components of protein complexes via a biotinyl or lipoyl group which is

covalently bound to a highly conserved lysine residue usually flanked by methionine residues (307, 308). Here, the homologous conserved MK signature (residues 259 and 260) which was predicted in this part of Alg44 and other highly conserved periplasmic residues were assessed for their impact on acetylation and epimerisation of alginate using alanine substitutions.

Compositional analysis of alginates showed the replacement of these residues with alanine decreased acetylation degree to 34%-52% when compared to of the control expressing *alg44* in cis (acetylation = 64%) (Table 1 and 2). Among mutants, M259A and D268A showed the highest impact on acetylation degree with 38% and 34%, respectively. Alanine substitution of these residues also gave the lowest value of alginate yield (Table 1 and 2).

Additional copy of Alg44 contributed to lower molar fraction of G residues (F_G) = 0.14 which was consistent with previous results. Interestingly, S265A did not change molar fraction of G residues while substitutions of other residues increased molar fraction of G residues to 0.2-0.23 and M259A showed the highest epimerisation degree (F_G) = 0.3 (Table 1 and 2). These data demonstrated that the periplasmic domain of Alg44 cannot directly mediate acetylation pathway, but has significant influence on regulation of modifications, boosting acetylation rather than epimerisation.

Table 1- Composition of alginates produced by different variants of Alg44

	Ac%	F_M	F_G	$F_{MG/GM}$	F_{MM}
300+MCS5	52	0.6	0.4	0.4	0.2
$\Delta 44+44his$	64	0.86	0.14	0.14	0.72
Q258A	50	0.8	0.2	0.2	0.6
M259A	38	0.7	0.3	0.3	0.4
K260A	49	0.8	0.2	0.2	0.6
T264A	52	0.78	0.22	0.22	0.56
S265A	49	0.86	0.14	0.14	0.72
D268A	34	0.77	0.23	0.23	0.54

300:PDO300; MCS5: pBBR1MCS-5; F_G : molar fraction of guluronate (G) residue; F_M : molar fraction of mannuronate (M) residue; $F_{GM/MG}$: molar fraction of two consecutive G and M residues; F_{MM} : molar fraction of two consecutive M residues; Ac.: acetylation

Table 2- Descending order of values presented in Table 1

Mutant	F_G	Mutant	Ac%	Mutant	F_{MM}
300+MCS5	0.4	$\Delta 44+44his$	64	$\Delta 44+44his$	0.72
M259A	0.3			S265A	0.72
D268A	0.23	300+MCS5	52	Q258A	0.6
T264A	0.22	T264A	52	K260A	0.6
Q258A	0.2	Q258A	50	T264A	0.56
K260A	0.2	K260A	49	D268A	0.54
		S265A	49		
$\Delta 44+44his$	0.14	M259A	38	M259A	0.4
S265A	0.14	D268A	34	300+MCS5	0.2

300:PDO300; MCS5: pBBR1MCS-5; F_G : molar fraction of guluronate (G) residue; F_{MM} : molar fraction of two consecutive M residues; Ac.: acetylation

Heterologous and homologous production of Alg44 and purification

Overproduction and purification of membrane-anchored proteins is challenging (309). The first challenging step is to express encoding genes of these proteins in commonly used heterologous hosts such as *Escherichia coli* laboratory strains. Among identified subunits constituting alginate biosynthesis multiprotein complex, some, including AlgG (26), AlgX (24), AlgK (19, 310), AlgE (62), AlgL (311), have been heterologously overproduced and purified for further analyses such as *in vitro* enzymatic assays and X-ray crystallography. To our knowledge overproduction of two cytoplasmic membrane-anchored proteins Alg8 and Alg44 (full-length) has not been successfully achieved as yet. Only catalytic domain of Alg8 and recently the soluble cytoplasmic domain of Alg44 (i.e. PilZ domain) were heterologously overproduced (59, 296) and the latter was purified for crystallization (296).

Here, we assessed the capability of different *E. coli* strains to expression full-length *alg44-12His* gene. Strains C41(DE3) and C43(DE3) did not show any detectable gene expression and BL21(DE3) showed only weak expression while Origami™ (DE3), ClearColi® and Rosetta™ strains gave rise to high expression levels of the *alg44-12His*

gene (Fig. 6). However, a significant fraction of protein was subjected to proteolytic degradation during membrane isolation, even in the presence of cocktail protease inhibitors. The strain Origami™ (DE3) (pETDuet-1:*alg44*-12His) was selected for scaling up protein production, followed by protein purification.

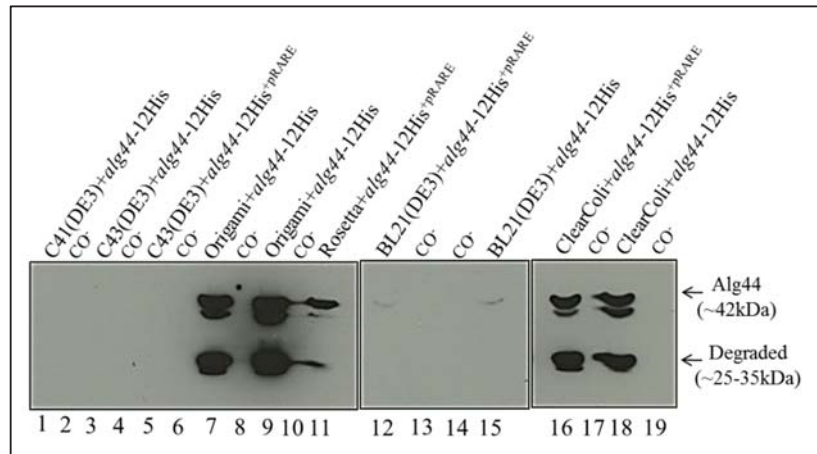


Fig. 6. Assessment of heterologous production of Alg44. Immunoblot of the envelope fractions of different *E. coli* strains developed using anti-His-tag antibody showed that Alg44 was produced by some *E. coli* strains including Origami (lanes 7 and 9) and ClearColi (lanes 16 and 18) strains while proposed strains for membrane protein production failed (lanes 1, 3, 5, 12 and 15). However, produced Alg44 was highly proteolytically degraded or truncated. All genes were carried by pETDuet-1 vector. Negative controls harbored empty plasmids. Co⁻: negative control.

An optimal purification of Alg44 was not obtainable because of degradation/truncation and protein contamination. Utilizing high and low ionic strength of buffers in addition to applying cocktail protease inhibitors and EDTA 5mM did not improve this procedure, however partial purification might be achievable by employing other methods of purification (Fig. 7). Therefore, homologous overproduction of Alg44 in *P.aeruginosa* was assessed (although it was initially thought not being ideal because additional copies of Alg44 led to overproduction of alginate which impaired bacterial growth). For this reason the mutant PDO300 Δ alg44 complemented with the *alg44*-6His integrated as a single copy into the genome was applied for Alg44 production and purification. This strategy was chosen to avoid the alginate overproduction. Importantly, initial protein analysis using immunoblotting showed that utilization of 5mM EDTA significantly reduced proteolytic degradation of Alg44 (Fig. 7). Membrane fraction of disrupted cells (7 g of wet cell mass) was subjected to affinity purification in the presence of a cocktail of protease inhibitors, 5mM EDTA and DTT. Interestingly, two distinct bands corresponding to the molecular mass of Alg44 monomer and dimer were detected using immunoblotting (Fig. 7).

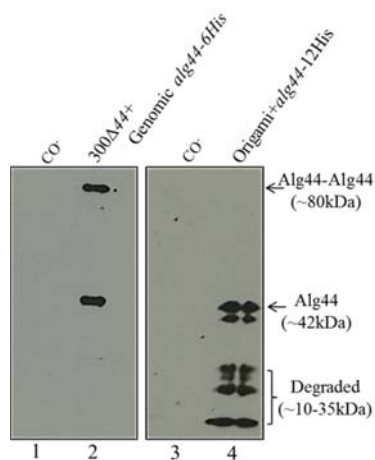


Fig. 7. Assessment of Alg44 purification produced from *P. aeruginosa* (left) and *E. coli* (right) hosts and treated with EDTA. Immunoblots showed the preliminary assessment of Alg44 purification for optimizing purification procedure and assessing EDTA effect on degradation. Immunoblots were developed using anti-His-tag antibody. Immunoblot of heterologous-based purification procedure showed that Alg44 was highly degraded in the presence of EDTA (lane 4) while degradation did not happen for homologous production when it was EDTA-treated (lanes 2). Negative controls harbored empty plasmids. 300: PDO300; MCS5: Co⁻: negative control.

To further purify Alg44, size exclusion chromatography was employed. The chromatogram showed two major peaks (Fig. 8). Interestingly, when protein samples

were treated with 0.2% alginate solution and DTT, impurities were dissociated from two major peaks while they were still impure without treatment with DTT or alginate solution (Fig. 8). Protein analysis of this purification step showed the first major peak (Fig. 8B, Peak I) belonged to the dimer of Alg44 (~85 kDa) which was mainly collected in two 500- μ l fractions (with protein concentration of 2.88 and 2.92 mg/ml respectively in the first and second fraction), while the second peak corresponding to monomer was not detectable (Fig. 8). Replacement of triton X-100 with DDM caused the loss of the stability of dimer along with protein degradation (Fig. 8). Protein sequencing of purified band confirmed Alg44 presence (coverage 67.61%; unique peptides: 24) along with the presence of the proposed signal peptide sequence (Fig. 8). Besides achieving full-length Alg44 purification, these experiments informed this protein has a very stable dimer form in solution, therefore the treatment with DDT did not disrupt the dimer. This experiment suggested Alg44 dimerisation was not dependent on disulfide bond formation. This result was confirmed previous finding suggesting dimerisation of full-length Alg44 (295) and is consistent with dimer of the purified PilZ_{Alg44} (296).

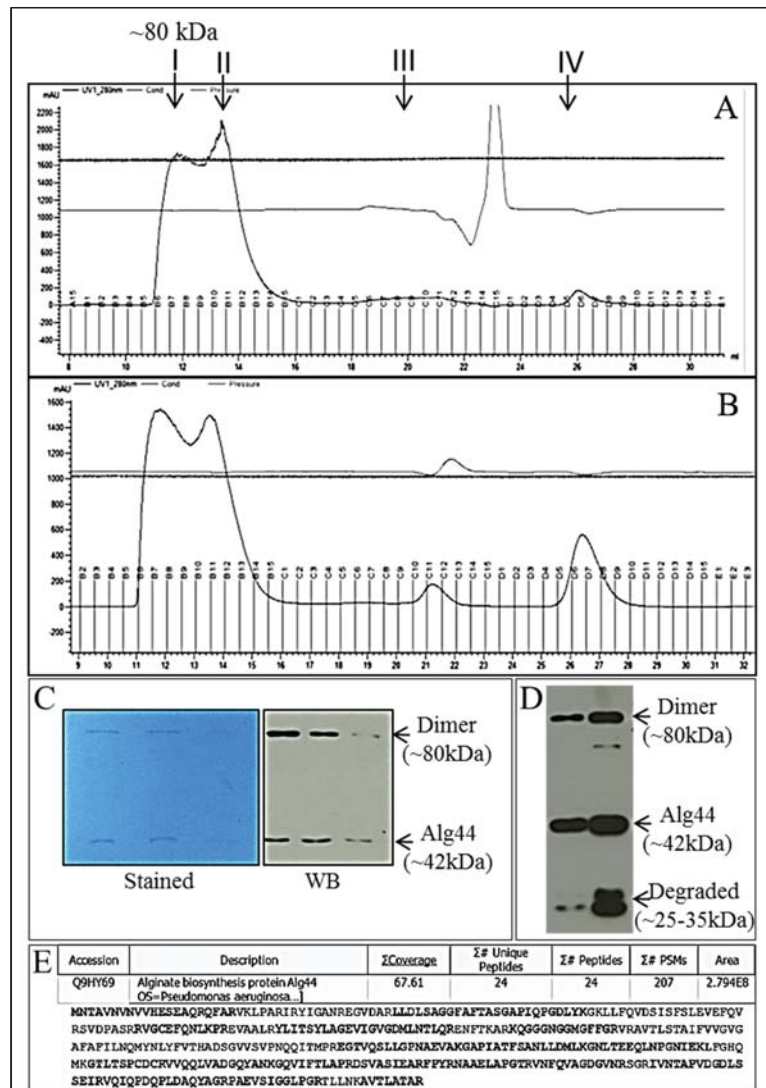


Fig. 8. Purification of Alg44 dimer using gel filtration chromatography. (A-B) Chromatograms belong to purification of Alg44-6His from a partially purified sample (provided using His-tag affinity chromatography), before (A) and after (B) treatment with alginate solution and DTT. Two major peaks I and II were separated. Presumable impurities associated with these peaks were removed after treatment of protein sample with 0.2% alginate solution and 50 mM DTT for overnight and resulted in peaks III and IV appearance. Protein analysis using immunoblotting showed peak I which was collected in three 500- μ l fractions contained Alg44 dimer (C). Protein concentration in each fraction of peak I was quantified as 2.88-2.99 mg/ml. Peak II was not detectable and identifiable using SDS-PAGE staining or immunoblotting. (C) SDS-PAGE gel (stained with Coomassie Brilliant Blue) (left) and immunoblot (right) of peak I showed dimer and monomer bands of Alg44. Immunoblots were developed using anti-His-tag antibody. (D) Immunoblot showed that treatment of sample with n-Dodecyl β -D-maltoside (DDM) during purification resulted in loss of Alg44 dimer stability and more truncations. (E) Protein sequence of purified band showed the existence of Alg44 with its signal peptide. Bold letters indicate identified peptides by mass spectrometry.

Discussion

In this study, we investigated the periplasmic domain of Alg44 using site-specific mutagenesis approaches. We assessed the impact of alanine substitution of some highly conserved periplasmic residues (Fig. 1) on alginate production, polymerisation, composition and protein stability. These residues are clustered in a region of the periplasmic domain which was bioinformatically predicted for having specific folding similar to biotinyl-lipoyl-domains of biotin/lipoyl attachment proteins with carboxylase/decarboxylase and acyltransferase activity (302). The overproduction of Alg44 in heterologous and homologous hosts was assessed and the purification of this protein was also demonstrated. This study revealed replacement of each residue caused a significant reduction or the abolishment of alginate production (Fig. 2) by impacting on polymerisation process (Fig. 3) or protein stability (Fig. 4). The cluster of PCDC (residue 266-269) was identified for being critical for Alg44 stability in the periplasm (Fig. 4). The role of CXC motif on stability of other proteins such as outer membrane lipoprotein CsgG within the periplasm of bacteria and copper chaperones in mitochondrial intermembrane space and its functional importance for copper trafficking by CopC protein across bacterial periplasm have been reported (304-306). In Alg44 both cysteine residues were required for protein stability. The role of these residues for intra- and intermolecular interactions is still not clear, but evidence is provided for a potential involvement in protein-protein interaction when Alg44 existed in native stoichiometry (Fig. 5).

This study suggested the impact of the periplasmic domain of Alg44 on alginate modification events. Consistent with previous results which showed Alg44 boosts acetylation of alginate (295), here replacement of periplasmic residues impaired the boosting effect on acetylation while causing a higher level of epimerization (Tables 1 and 2). These data suggested a regulatory effect of Alg44 on modifications of alginate through periplasmic domain.

In the present study, we attempted full-length Alg44 production and purification. We showed that overproduction of Alg44 by *E. coli* strains was achievable, but not by those designed for production of membrane proteins, such as C41 (DE3) and C43(DE3) (Fig. 6). The best production host was *P. aeruginosa* containing the *alg44-6His* gene integrated into the genome. Purification of an Alg44 dimer with high stability was achieved when EDTA was added and protein sample was treated with alginate solution and DTT to remove impurities (Fig. 7 and 8). These results confirmed Alg44

dimerisation which was already suggested on the basis of crosslinking experiments (295). Recently, the dimer form of cytoplasmic PilZ_{Alg44} domain was demonstrated in x-ray crystallography of this domain (296).

Acknowledgments

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Final Discussion and outlook

Discussion

Bacteria produce a wide range of polymeric substances. Bacterial polysaccharides are among the most diverse biopolymers. Each polysaccharide has a specific biological function relevant to the producer microorganism. The majority of polysaccharides are synthesized and secreted into the environment and referred to as exopolysaccharides. Their chemical structures can be homopolymeric or heteropolymeric, showing a range of glycosidic linkages and molecular weights, contributing to various material properties. Their production is an advantage for bacteria to survive under harsh conditions; alternatively, they are involved in the pathogenicity process. Biofilm formation is a survival strategy for bacteria where they attach to surfaces and initiate cellular aggregations and production of complex extracellular polymeric substances (EPS) mainly composed of exopolysaccharides. These bacteria can develop biofilm communities in a wide range of environments such as soil, water, living tissues and any moist surface exposed to nutrient flow (312). Biofilm formation increases bacterial resistance to bacteriophages, biocides, antibiotics, toxic materials, immune response, harsh environments and mechanical stresses (270, 312).

Cellulose, alginate, xanthan, dextran and complex capsular polysaccharides are well-known bacterial exopolysaccharides (313, 314). For a long time, they have been studied for unravelling their biological function, biosynthesis pathways and potential industrial applications. Understanding biosynthesis pathway of bacterial exopolysaccharides is tremendously important for development of drugs against pathogenic bacteria producing exopolysaccharides involved in pathogenesis. They can also be harnessed in numerous medical and industrial applications via genetic engineering towards tailor-made production (314).

Bacterial exopolysaccharides are produced via different biosynthesis pathways where the genes encoding the enzymes and secretion apparatus are usually clustered within the genome of respective production bacteria.

Until now, four general mechanisms have been known in bacteria by which exopolysaccharides are produced: (i) the so-called Wzx/Wzy-dependent pathway; (ii) the ATP-binding cassette (ABC) transporter dependent pathway; (iii) the synthase-dependent pathway; and (iv) the extracellular synthesis by use of a single sucrose protein (314).

In all pathways other than (iv) exopolysaccharide polymerisation/secretion is performed by multiprotein complexes localized in the cell envelope of bacteria, while precursor biosynthesis occurs in the cytoplasm (314). Polymerisation mainly consists of polymerising active precursors into exopolysaccharide by a single enzyme (i.e. glycosyltransferase) or a multi-enzyme catalytic unit, translocation of polymer across cytoplasmic membrane and periplasm, probable modifications and export across outer membrane for secretion.

Alginate and cellulose syntheses follow the synthase-dependent pathway, where complete polysaccharide chain is secreted across bacterial outer membrane and cell wall (314). The synthesis of these two polysaccharides is performed by different envelope-spanning multiprotein complexes.

Alginate is a virulence factor during infection by *P. aeruginosa*, particularly in cystic fibrosis patients. *P. aeruginosa* has been a model for alginate biosynthesis not only due to its importance in pathogenesis and biofilm formation, but also due to its material properties for numerous industrial applications. This study focused on understanding the molecular mechanisms underlying alginate polymerisation and modification.

Despite many approaches in unravelling protein functions and structures involved in bacterial exopolysaccharide biosynthesis, many aspects of them have been unknown yet. This thesis provides insights into the arrangement and assembly of the alginate biosynthesis/modification/secretion multiprotein complex spanning the envelope of *P. aeruginosa* (Chapter IV and V), interactive functional relationship of different subunits constituting multiprotein complex, mechanism of polymerisation activation and correlation of alginate polymerisation and modification mechanisms (Chapter V).

The synthesis of alginate, cellulose and poly-N-acetylglucosamine (PNAG) is post-translationally regulated by the second messenger c-di-GMP which controls a wide range of biological processes in bacteria through unknown mechanisms. Recently, the first crystallographic snapshot of cellulose synthesis in association with c-di-GMP was provided and the mechanism of cellulose polymerisation activation was studied. Since cellulose and alginate synthases share similar organisations, here it was suggested that alginate polymerisation activation resembles cellulose polymerisation activation (270, 315). Experimental strategies included bioinformatic-based structural modelling and site specific mutagenesis were used to analyse activation mechanism of alginate polymerisation. The activating effector c-di-GMP was shown to bind to the PilZ domain of Alg44, causing activation of polymer synthesis, presumably implying a different

molecular mechanism for alginate and cellulose (Chapter V). This was further supported by a recent study by Whitney et al. (2015) which showed that the dimeric form of c-di-GMP binds to PilZ_{Alg44} domain required for alginate polymerisation, while a monomeric c-di-GMP is involved in cellulose synthase activation (296). However, that report did not show mechanism underlying alginate polymerisation activation. This thesis showed that alanine substitutions of R residues in the PilZ_{Alg44} RXXXXR motif that binds c-di-GMP abolished alginate production under both permissive and non-permissive condition (i.e absence and presence of RocR protein which degrades c-di-GMP, respectively) (Chapter V). This supports the necessity of PilZ_{Alg44} domain conformational change upon binding to c-di-GMP for alginate polymerisation. It is not clear yet if this conformational change breaks any possible steric clashes towards opening up proposed gating loop and then the catalytic site or other events occur. We showed, however, that under non-permissive conditions H323A mutation of Alg8 increased the activity of alginate synthase. This residue was chosen as a candidate for forming a salt bridge with R residues of RXXXXR motif of Alg44 because the position of this residue was found to be identical to salt bridge forming residue of BcsA, i.e. E460, in pairwise alignment of Alg8 and BcsA. But the combination of Alg8(H323A), Alg44(R17A and R21A) did not activate alginate polymerisation. Additionally, it was proposed that in cellulose synthase activation, resting state of gating loop may be necessary for loading precursor onto catalytic site. The relationship of conformational change of PilZ_{Alg44} and modeled gating loop of Alg44 is unclear and investigation of this mechanism is in progress.

Modifications of alginate in *P. aeruginosa* include epimerisation of M to G residues and acetylation, both of which impact the physiochemical properties of alginate. Particularly, this is important in order to achieve ability to produce microbial alginate that have defined properties suitable for specific applications.

Even though alginate modification was discovered a long time ago, the relationship between alginate polymerisation and modification had not been investigated. This study showed that alginate polymerisation and modifications are linked. The molecular mass of alginate was reduced by epimerisation, while it was increased by acetylation, indicating epimerisation, but not acetylation interferes with processivity of alginate polymerisation (Chapter V). Fundamental physiological roles of polysaccharides are dependent on their length and the mechanisms of length control by biosynthetic enzymes are poorly understood (316). In the synthesis of other exopolysaccharides such

as xanthan and capsular polysaccharides the mechanism of polymer length control is mediated by specific proteins (e.g. polysaccharide co-polymerases) which uncouple the synthesis of the polymer from the polymerisation unit (275, 317). This study provided the first experimental evidence that showed the interplay of molecular weight of alginates with modification mechanisms via impacting the processivity of polymerase. However, the involvement of alginate lyase AlgL in controlling alginate length cannot yet be ruled out.

Although the function of alginate epimerase AlgG and acetyltransferase AlgX were characterised on the basis of *in vivo* and *in vitro* enzymatic assays and crystallographic structures, the interactive roles of these proteins were unknown. In this study, generation of double-gene knockout mutants in *algG* and *algX* genes and complementation by active and inactive variants of relevant proteins enabled us to reveal the functional relationship of epimerisation/acetylation and AlgG/AlgX proteins. Here, epimerisation and acetylation were found not to compete with each other, but were linked. Furthermore, AlgG (epimerase) and AlgX (acetyltransferase) were found to enhance each other's functions (Chapter V). Alg44 was found to be another member in the multiprotein complex which was not only necessary for polymerisation, but also regulates modification events (Chapter V and VI). This study and the recent finding of dimeric c-di-GMP binding to Alg44 showed that it is a unique protein. Alg44 is proposed to function as alginate co-polymerase due to binding to dimeric c-di-GMP with its cytoplasmic domain. The periplasmic domain was proposed to be involved in activation of alginate polymerase (Alg8) through direct interaction and regulation of alginate modification events via interreaction with AlgX and AlgK.

Outlook

For many years, *P. aeruginosa* has been studied as a model organism for understanding of alginate biosynthesis. The structures and functions of many proteins which are directly involved in alginate biosynthesis have been determined and the existence of an envelope-spanning multiprotein complex was proposed. However, assembly and arrangement of proteins within the complex and their functional relationships were unclear. A report by Hay et al. in 2012 (279) was first to demonstrate protein-protein interactions and AlgK-AlgX-MucD network. We identified more periplasmic protein complexes; AlgK-AlgE, AlgK-Alg44, AlgX-Alg44 and Alg44-Alg44 dimer have now been identified in this thesis. Also, this study identified direct interaction of two

cytoplasmic membrane proteins, Alg8 and Alg44, which form alginate polymerisation unit of this multiprotein complex. However, exact superimposition of all these interacting proteins and involved domains are still unknown.

Proposed multiprotein complex is linked to different post-translational mechanisms for the regulation of alginate production. Many years ago it was understood that alginate biosynthesis is essentially dependent on c-di-GMP, a ubiquitous bacterial second-messenger molecule which was found to bind PilZ_{Alg44}, however, the role of c-di-GMP in alginate synthesis was unknown. This study showed that c-di-GMP is required for activation of polymerisation upon binding to alginate synthase complex, in a mechanism which was different from activation of cellulose synthase. However, structural mechanism behind this activation remains to be determined. Recently, Whitney and co-workers have crystallized PilZ_{Alg44} domain and they found that a unique dimeric form of c-di-GMP is required for alginate biosynthesis (296).

Although epimerisation and acetylation of alginate had been discovered decades ago, the functional relationship of these events and relevant proteins and their interplay with alginate polymerisation unit was unknown. This study showed that these events were uncompetitive and linked and also they link to polymerisation process. We showed that the correlation of molecular mass of alginate with modifications. Also, we showed that AlgX and AlgG can enhance each other's functions. However, how superimposition of these proteins allows such an interplay and coordination is still a question.

Even though many proteins of proposed multiprotein complex have been crystallized and their structure has been elucidated, the high resolution structure of polymerase synthase has not been resolved because production and purification of Alg8 and Alg44 under native conditions have not been successful as yet. Here, we demonstrated purification of full-length Alg44 from *P. aeruginosa*. This study showed the existence of Alg44 in solution as a first step towards crystallisation. However, the preparation of purified protein for being ideal for crystallization is a challenge which needs new efforts to resolve.

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