Polymerisation and export of alginate in *Pseudomonas aeruginosa*: Functional assignment and catalytic mechanism of Alg8/44

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Preface

This thesis is based on the latest version (DRC 06/139 from 31/08/2006) of the "Submission of a thesis based on publications" format as described on page 53 of the "Handbook for Doctoral Study" of Massey University.

The following parts and chapters of this thesis have been published or are submitted for publication in internationally peer-reviewed journals. The publications used in this thesis do not appear in chronological order.

Chapter I (General introduction)

Uwe Remminghorst and Bernd H. A. Rehm (2006). Bacterial alginates: from biosynthesis to applications. Biotechnology Letters **28** (21), 1701-1712.

Chapter II

Uwe Remminghorst and Bernd H. A. Rehm (2006). *In vitro* alginate polymerisation and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. Applied and Environmental Microbiology **72** (1): 298-305.

Chapter III

Uwe Remminghorst and Bernd H. A. Rehm (2006). Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. FEBS Letters **580** (16): 3883-3888.

Chapter IV

Uwe Remminghorst and Bernd H. A. Rehm (2007). Membrane topology and site-specific mutagenesis of Alg8, a putative glycosyltransferase involved in alginate polymerisation by *Pseudomonas aeruginosa*. Journal of Bacteriology – submitted for publication.

Chapter V

Andrea Campisano, Zoe Jordens, Uwe Remminghorst and Bernd H. A. Rehm (2007). Attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa*. Journal of Bacteriology - submitted for publication.

Chapter VI

Jana Gutsche, Uwe Remminghorst and Bernd H. A. Rehm (2006). Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie 88 (3-4): 245-251.

The following experiments of the publications/chapters were performed by Uwe Remminghorst:

Chapter I: The review article was mainly written by Uwe Remminghorst.

Chapter II: The *in vitro* alginate polymerisation assay, including the enzymatic synthesis and purification of GPD-mannuronic acid, and the development of the threading model was performed by Prof. Bernd H. A. Rehm. Prof. Gudmund Skjåk-Bræk performed the ^{1}H-NMR analyses of the alginate samples. All other experiments described in this chapter were performed by Uwe Remminghorst.

Chapter III: All experiments were performed by Uwe Remminghorst. As stated in the acknowledgement section of the publication, the proteomic analysis (MALDI-TOF/MS) was performed by Dr. Simone König (University of Münster, Germany) and the electron microscopy analysis was performed by Mr. Aaron Hicks (Massey University, New Zealand).

Chapter IV: All experiments were performed by Uwe Remminghorst.

Chapter V: The PHA content analysis of the different strains was performed by Uwe Remminghorst.

Chapter VI: All primer and the cloning strategy for the knockout generation were designed by Uwe Remminghorst. The construction of the β-galactosidase and alkaline phosphatase fusion constructs and their respective reporter gene assays were performed by Uwe Remminghorst.

This is to certify that the above mentioned experiments and/or tasks have been conducted by Mr. Uwe Remminghorst.

29/06/07 _____

(Date, Signature)

Prof. Dr. Bernd H. A. Rehm

29/06/07 _____

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Uwe Remminghorst

Abstract

Alginate biosynthesis is not only a major contributor to pathogenicity of *P. aeruginosa* but also an important factor in colonization of adverse environmental habitats by biofilm formation. The requirement of proteins Alg8 and Alg44, encoded by their respective genes in the alginate biosynthesis gene cluster, for alginate biosynthesis of *P. aeruginosa* was demonstrated, since deletion mutants were unable to produce or polymerise alginate. AlgX deletion mutants failed to produce the alginate characteristic mucoid phenotype, but showed low concentrations of uronic acid monomers in the culture supernatants. Complementation experiments using PCR based approaches were used to determine the complementing ORF and all deletion mutants could be complemented to at least wildtype levels by introducing a plasmid harbouring the respective gene. Increased copy numbers of Alg44 did not impact on the amount of alginate produced, whereas increased copy numbers of the *alg8* gene led to an at least 10 fold stronger alginate production impacting on biofilm structure and stability. Topological analysis using reporter protein fusions and subsequent subcellular fractionation experiments revealed that Alg8 is located in the cytoplasmic membrane and contains at least 4 transmembrane helices, 3 of them at its C terminus. Its large cytosolic loop showed similarities to inverting glycosyltransferases and the similarities were used to generate a threading model using SpsA, a glycosyltransferase involved in spore coat formation of *B. subtilis*, as a template. Site-directed mutagenesis confirmed the importance of identified motifs commonly detected in glycosyltransferases. Inactivation of the DXD motif, which has been shown to be involved in nucleotide sugar binding, led to loss-of-function mutants of Alg8 and further replacements revealed putative candidates for the catalytic residue(s). Contradicting the commonly reported prediction of being a transmembrane protein, Alg44 was shown to be a periplasmic protein. The highest specific alkaline phosphatase activity of its fusion protein could be detected in the periplasmic fraction and not in the insoluble membrane fraction. Bioinformatical analysis of Alg44 revealed structural similarities of its N terminus to PilZ domains, shown to bind cyclic-di-GMP, and of its C terminus to MexA, a membrane fusion protein involved in multi-drug efflux systems. Thus, it was suggested that Alg44 has a regulatory role for alginate biosynthesis in bridging the periplasm and connecting outer and cytoplasmic membrane components. AlgX was shown to interact with MucD, a periplasmic serine protease or chaperone homologue, and is suggested to exert its impact on alginate production via MucD interaction. *In vitro* alginate polymerisation assays revealed that alginate production requires protein components of the outer and cytoplasmic membrane as well as the periplasm, and these data were used to construct a model describing a multi-enzyme, membrane and periplasm spanning complex for alginate polymerisation, modification and export.

Table of contents

Chapter II:

Chapter III:

Chapter IV:

Chapter V:

Chapter VI:

Chapter VII:

List of tables

Chapter II:

Chapter III:

Chapter IV:

Chapter VI:

List of figures

Chapter I:

Chapter II:

Chapter III:

Chapter IV:

Chapter V:

Chapter VI:

Chapter VII:

Abbreviations

A.	*Azotobacter*
°C	Degrees Celsius
C or Cys	Cysteine
c-di-GMP	bis-(3'-5')-cyclic dimeric guanosine monophosphate
CM	Cytoplasmic membrane
CoA	Coenzyme A
D or Asp	Aspartate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease A
E or Glu	Glutamate
E.	*Escherichia*
EPS	Exopolymeric substance
Fig.	Figure
G	Guluronic acid
GDP	Guanosine 5'-(trihydrogen diphosphate)
GFP	green fluorescent protein
GT	Glycosyltransferase
GTP	Guanosine triphosphate
H or His	Histidine
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethane sulfonic acid]
HMM	Hidden Markov model
K or Lys	Lysine
kDa	Kilo Dalton
KDO	2-Keto-3-deoxyoctanoate
LacZ	β-Galactosidase
LB	Luria-Bertani
M	Mannuronic acid
MALDI-TOF	Matrix-assisted laser desorption ionisation time-of-flight
MS	mass spectrometry
Mw	Molecular weight
$NAD^+[H]$	Nicotinamide adenine dinucleotide [reduced form]
ND	not detectable
NMR	Nuclear magnetic resonance
OD	Optical density
OM	Outer membrane
ORF	open reading frame
P.	*Pseudomonas*
PAGE	Polyacrylamide gel electrophoresis
PhoA	Alkaline Phosphatase
PIA	*Pseudomonas* isolation agar
PIM	*Pseudomonas* isolation medium
PronaseE	Proteinase E
RNase	Ribonuclease A
SDS	Sodium dodecyl sulphate
TCA	Tricarboxylic acid cycle
TLC	Thin layer chromatography
TM	Transmembrane

Chapter I

General introduction

Bacterial extracellular polysaccharides and their biosynthesis

Bacterial exopolysaccharides

Numerous bacteria produce extracellular polysaccharides (exopolysaccharides), i.e. polysaccharides found outside the bacterial cell, either attached to it in form of capsules or secreted into the extracellular environment in form of mucus or slime. Such polymers vary considerably in their chemical structures as well as in the way they are synthesized by their bacterial hosts. Some components such as D-glucose, D-mannose, D-galactose and D-glucuronic acid occur very frequently in different exopolysaccharides, others like L-rhamnose or L-fucose are slightly less common, and D-mannuronic acid or L-guluronic acid are rarely seen as polymer constituents.

Generally, exopolysaccharides can be classified into 5 groups. The first group comprises dextran and the related polysaccharides "mutans" or levans, which are produced by species of the genera *Streptococcus* and *Leuconostoc*. These polymers consist entirely of one monosaccharide type (homopolysaccharides) and are in general produced by bacteria utilizing sucrose as a specific substrate. In absence of the specific substrate, the organisms can grow but are unable to form the polysaccharide. The second group is closely related to the first one, but in contrast to homopolysaccharides like levans or dextrans, a heteropolysaccharide is produced, i.e. a polysaccharide containing more than one component monosaccharide. There are a few organisms reported to produce heteropolymers only when metabolizing a specific carbon substrate, one of them is *Bacillus licheniformis*, which forms a polymer containing D-glucose, D-mannose and D-xylose (Ebube et al. 1992). The third group consists of exopolysaccharides being produced in form of homopolysaccharides by a number of microorganisms utilizing a variety of different carbon sources. The polymer is usually formed when the cytosolic precursor transits the cytoplasmic membrane or traverses the periplasm, and is followed by a secretion or export process of the nascent chain. Some of these homopolymers are solely composed of carbohydrates, e.g. bacterial cellulose or pullulan, others contain additionally functional groups like acyl groups (succinyl and pyruvyl substituents).

Probably the largest, but certainly the most heterogeneous group of exopolysaccharides is composed of those formed from repeating block structures. Most are originated from relatively simple structural units ranging in size from disaccharides to octasaccharides, but other components like acyl groups may also be present. A representative of this group is the exopolysaccharide xanthan produced by *Xanthomonas campestris*. This acidic heteropolysaccharide consists of D-glucosyl, D-mannosyl, and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of O-acetyl and pyruvyl residues. Alginate represents the fifth and last group of exopolysaccharides. This group contains heteropolysaccharides composed of two types of

monomers (e.g. alginate with D-mannuronic acid and L-guluronic acid), together with various side groups, like the O-acetyl groups of alginate. However, unlike the heteropolysaccharides of the previous group, there is no repeating unit. The polymer molecule contains a random monomer composition which also enables the formation of block sequences consisting of only one monomer. These different block formations interspersed by random monomer sequences give polymers like alginate a variety of different chemical properties, even enabling the design of special "tailor-made" polymers providing unique features.

The following section tries to give a brief introduction into 3 different bacterial exopolysaccharides and their biosynthesis pathways and key enzymes. It will focus on the bacterial exopolymers dextran, xanthan and cellulose, which are not only key representatives of the different groups but are also the most studied examples and are of commercial interest. A more detailed analysis of alginate, its chemical characteristics and its biosynthesis is given in the following chapter (Chapter I – Bacterial Alginates).

Dextran

Dextrans, or α-D-glucans, are defined as homopolysaccharides of glucose that feature a substantial number of consecutive α(1-6) linkages in their major chains. Normally those linkages make up more than 50% of total polymer linkages. Dextrans can possess side chains originating from α(1-2), α(1-3), or α(1-4) branch linkages, but the exact molecular structure of each dextran is closely associated to its specific bacterial production strain. Dextrans are produced by a number of lactic acid bacteria, in particular strains of *Streptococcus* species and *Leuconostoc mesenteroides* (Cerning 1990; Iliev et al. 2001). Because dextrans are primarily formed from sucrose, they have been known as contaminants of food products and sugar refineries. Since dextran-producing bacteria do not break down the polymers, dextrans presumably do not serve as storage materials (Leathers 2005). It is suggested that these polysaccharides serve to protect cells from desiccation or help them to adhere to environmental substrates.

In contrast to almost all other exopolysaccharides, dextrans are produced extracellularly by secreted enzymes commonly referred to as glucansucrases, or more precisely, dextransucrases. These Ca^{2+}-dependent enzymes represent glycosyltransferases that catalyze the transfer of the D-glucopyranosyl subunits from sucrose to dextrans, whereby fructose is released by the enzyme and consumed by growing cells. Neither adenosine 5'-triphosphate (ATP) nor other cofactors are required for these reactions, as the enzyme utilizes energy available in the glycosidic bond between glucose and fructose (Leathers 2005). The glucansucrase genes of different bacterial hosts appear to be closely related and their encoded proteins exhibit a common organizational

structure, with a conserved N-terminal catalytic domain and a C-terminal glucan-binding moiety that contains a series of direct tandem repeats (Monchois et al. 1999; Remaud-Simeon et al. 2000). Potentially important catalytic residues in these glucansucrases have been proposed by a number of authors based on consensus sequences and site-directed mutagenesis experiments (Arguello-Morales et al. 2000; Monchois et al. 2000a; Monchois et al. 2000b; Remaud-Simeon et al. 2000).

Xanthan

Xanthan is a complex exopolysaccharide produced by the plant-pathogenic bacterium *Xanthomonas campestris* pv. campestris. The polymer consists of D-glucosyl, D-mannosyl, and D-glucuronyl acid residues and variable proportions of O-acetyl and pyruvyl residues. Those residues form an acidic polymer build of pentasaccharide subunits, which form a cellulose backbone with trisaccharide side-chains composed of mannose-β(1-4)-glucuronic acid-β(1-2)-mannose attached to alternate glucose residues in the backbone by α(1-3) linkages. Approximately half of the terminal mannose residues carry a pyruvic acid moiety joined by a ketal linkage. Its unique physical properties allow xanthan to be used in a variety of different applications, like as a thickener or viscosifier in both food and non-food industries. Xanthan is also used as a stabilizer for numerous suspensions, emulsions, and foams.

Unlike dextran, xanthan polymerization occurs at the cytoplasmic membrane transition of cytosolic activated sugar nucleotides, acetyl-CoA, and phosphoenolpyruvate. But in direct contrast to alginate and cellulose, which are also formed from activated sugar nucleotides, the precursors are transported across the inner membrane (cytoplasmatic membrane) by a polyisoprenol phosphate carrier (Ielpi et al. 1993). The polymerization involves a stepwise assembly of the repeating pentasaccharide and starts with the transfer of glucosyl-1-phosphate from UDP-glucose to a polyisoprenol phosphate, followed by the sequential addition of the other sugar residues to form the complete repeating unit. The acetyl and pyruvyl residues are added at the lipid-linked pentasaccharide level. Examination of the polymerization process itself indicated that the xanthan chain grows at the reducing end (Ielpi et al. 1993). The export mechanism of the polysaccharide is not yet fully understood but is thought to be associated to the polymerization event (Becker et al. 1998).

The genes involved in lipid-linked intermediate assembly, polymerization, and secretion have been isolated and sequenced. They are clustered in a 16-kbp region (Capage et al. 1987; Harding et al. 1987), called *xps1* or *gum*, and form an operonic structure containing 12 open reading frames (*gumB* to *gumM*) (Capage et al. 1987). Unlike other exopolysaccharide biosynthesis

operons this region is unlinked from those required for synthesis of the sugar precursor (Becker et al. 1997). Some of the proteins encoded by genes of the *gum* cluster could be functionally assigned. GumD was shown to catalyze the addition of glucose-1-phosphate to the polyisoprenol carrier, while GumM, GumH, GumK and GumI catalyze the sequential addition of the residual sugars of the pentasaccharide (Katzen et al. 1998). Other proteins like GumB, GumC, GumE, and GumJ showed accumulation of complete xanthan subunits *in vitro*, however complemented knock out mutants used in *in vivo* experiments failed to produce the polymer, hence their involvement in polymerization and/or export cannot be ruled out (Katzen et al. 1998).

Cellulose / Chitin

Cellulose is the most abundant biopolymer on earth, recognized as the key component of plant biomass, but it is also a representative of microbial extracellular polymers. A remarkable feature of bacterial cellulose (BC) lies in its ultrafine reticulated structure and its chemical purity. This distinguishes it from plant derived cellulose, which occurs usually associated with hemicelluloses and lignin. Cellulose is an unbranched polysaccharide formed of β(1-4) linked glucopyranose residues. The nascent chains aggregate to form subfibrils, which belong to the thinnest naturally occurring fibers possessing a width of approximately 1.5 nm (Iguchi et al. 2000). Cells of cellulose-producing bacteria (like *Gluconacetobacter xylinus*, formerly called *Acetobacter xylinum*) are entrapped in the polymer network, which helps the population to establish itself at the liquid-air interface (Williams and Cannon 1989). The polymer matrix also takes part in adhesion of cells onto any accessible surface and facilitates nutrient supply, since nutrient concentration in the polymer lattice is noticeably enhanced due to its adsorptive properties, when compared to the surrounding aqueous environment (Costerton et al. 1999).

Synthesis of BC is a precisely and specifically regulated multi-step process, involving a large number of both individual enzymes and complexes of catalytic and regulatory proteins, but their supramolecular structure has not yet been well defined. The synthesis of the cytosolic precursor uridine diphospho-glucose (UDP-Glc) is followed by the processive polymerization into a β(1-4)-glucan chain. These nascent chains associate into characteristic ribbon-like structures, formed by hundreds or even thousands of individual cellulose chains (Bielecki et al. 2005). Similar to the alginate biosynthesis, cellulose pathways and mechanisms of precursor synthesis are relatively well known, whereas molecular mechanisms of polymerization into long unbranched chains, their extrusion outside the cell, and their self-assembly into fibrils require further elucidation.

Throughout the genomes of cellulose producing bacteria, there are two genes always present in the cellulose biosynthesis operon, the cellulose synthase (*bcsA* – bacterial cellulose synthase) and the bis-(3',5') cyclic diguanylic acid (c-di-GMP) binding protein (*bcsB*) (Romling 2002). The cellulose synthase BcsA varies in length between species but normally comprises between 723 and 888 amino acids and is reported to be the most conserved gene of the bacterial *bcs* operon. Located in the cytoplasmic membrane, it contains 8 to 10 predicted transmembrane domains, and is considered to be the catalytic subunit for cellulose synthesis. In alginate biosynthesis, these features correspond to Alg8, which is also localized in the cytoplasmic membrane and is proposed to be the potential catalytic subunit (Remminghorst and Rehm 2006). The bacterial cellulose synthases contain a D,D,D35Q(R,Q)XRW motif, which is actually a characteristic of a whole group of processive β-glycosyltransferases including cellulose synthases as well as chitin and curdlan synthases (Romling 2002). This motif seems to be involved in binding of the nucleotide sugar precursor and is - in combination with other more cellulose synthase specific motifs - proposed to be part of the polymerization machinery. The free cytosolic secondary messenger c-di-GMP is considered to allosterically activate the cellulose synthase via protein BcsB (Romling 2002).

In *G. xylinus* the cellulose-synthesizing complex is a transmembrane complex spanning the cytoplasmic and outer membrane whereby the cellulose synthase and the c-di-GMP binding protein are considered to be localized in the cytoplasmic membrane (Kimura et al. 2001). In cells actively producing cellulose, approximately 50 cellulose-synthesizing multienzyme complexes are organized in a single row along the longitudinal axis of the bacterial rod. Each one of these complexes secret approximately 12 to 25 glucan chains which assemble into larger fibrils at the site of synthesis. This structure, called the linear terminal complex, can be visualized by electron microscopy as 35 Å pores in the outer membrane (Kimura et al. 2001).

Chitin is a β-aminoglucan and is formed as a linear polymer of β(1-4) linked *N*-acetyl-D-glucosamine residues. Rhizobia are soil bacteria and have established a symbiotic relationship with leguminous plants. This symbiosis results in the formation of a new plant organ, the root nodule, in which rhizobia are able to fix atmospheric nitrogen into biologically utilizable ammonia. One essential element triggering the nodulation process is the synthesis and secretion of the so called Nod factors, or lipo-chitin oligosaccharides (van der Drift et al. 1996). Most Nod factors contain a chitintetraose or –pentaose backbone, but Nod factors with a di-, tri-, or hexasaccharide backbone have also been detected (Olsthoorn et al. 1998). Although bacterial chitin production is limited to oligomeric levels, the oligosaccharide synthase, encoded by gene *nodC*, shows sequence similarity to the *Sacharomyces cerevisiae* chitin synthase (Bulawa 1992). As

indicated earlier, there is some degree of similarity between the chitin and cellulose synthases, which might be due to their precursor and polymer backbone similarity.

Similarities to Alg8, the putative alginate polymerase, are only based on secondary structure and are even more limited since precursor and polymer are different. Only the core feature the motif binding the activated sugar nucleotide (DXD motif), is comparable between Alg8 and cellulose or chitin synthases. But these structural motifs can also be found throughout class 2 glycosyltransferases.

Glycosyltransferases

An enormous amount of glycosidic-bond synthesis in nature is catalyzed by glycosyltransferases, which utilize activated glycosides as the sugar donor. Typically, the activated leaving group is a nucleoside phosphate, lipid phosphate or phosphate. The nucleotide-sugar-dependent glycosyltransferases are divided into over 50 sequence-based families, with the largest and most widespread family of inverting glycosyltransferases named family GT-2. In an inverting glycosyl transfer the product shows the opposite stereochemistry at the anomeric center than its substrate, in case of alginate the enzyme utilizes an α-activated D-sugar (GDP-mannuronic acid) giving rise to a β-D linked product (alginate). Mechanistically, glycosyltransferases are believed to proceed through an oxocarbenium-ion-like transition state similar to that proposed for glycosidases (Ünligil et al. 2000; Tarbouriech et al. 2001). The reaction is thought to involve general base catalysis and in many cases has been shown to depend on manganese (Mn^{2+}) ions (Murray et al. 1996; Ünligil et al. 2000).

Nucleotide sequences of many analyzed class-2-glycosyltransferases showed only limited similarity to each other, however, similarities could be detected based on secondary structure. In most cases those similar regions are domains where the activated sugar precursor is proposed to bind. A very conserved region, the so-called DXD motif, is proposed to be involved in the nucleotide binding process, which is thought to additionally require a manganese ion. Further insights into catalysis and structural similarities between different glycosyltransferases are given in chapters IV and VII.

References

Arguello-Morales, M. A., M. Remaud-Simeon, S. Pizzut, P. Sarcabal, R. Willemot and P. Monsan (2000). Sequence analysis of the gene encoding alternansucrase, a sucrose glucosyltransferase from *Leuconostoc mesenteroides* NRRL B-1355. FEMS Microbiol Lett **182**(1): 81-85.

Becker, A., F. Katzen, A. Pühler and L. Ielpi (1998). Xanthan gum biosynthesis and application: a biochemical/genetic perspective. Appl Microbiol Biotechnol **50**(2): 145-152.

Becker, A., S. Ruberg, H. Küster, A. A. Roxlau, M. Keller, T. Ivashina, H. P. Cheng, G. C. Walker and A. Pühler (1997). The 32-kilobase exp gene cluster of *Rhizobium meliloti* directing the biosynthesis of galactoglucan: genetic organization and properties of the encoded gene products. J Bacteriol **179**(4): 1375-1384.

Bielecki, S., A. Krystynowicz, M. Turkiewicz and H. Kalinowska (2005). Bacterial Cellulose. Biopolymers for Medical and Pharmaceutical Applications. A. Steinbüchel and R. H. Marchessault. Weinheim, Wiley-VCH.

Bulawa, C. E. (1992). *CSD2*, *CSD3*, and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol Cell Biol **12**(4): 1764-1776.

Capage, M. A., D. H. Doherty, M. R. Betlach and R. W. Vanderslice (1987). Recombinant-DNA mediated production of xanthan gum. Int patent **WO87/05938**.

Cerning, J. (1990). Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol Rev **7**(1-2): 113-130.

Costerton, J. W., P. S. Stewart and E. P. Greenberg (1999). Bacterial biofilms: a common cause of persistent infections. Science **284**(5418): 1318-1322.

Ebube, N. K., O. K. Udeala and A. A. Ghobashy (1992). Isolation and characterization of a novel polysaccharide from *Bacillus licheniformis* NCIB 11634. J Ind Microbiol Biotechnol **9**(3-4): 239-245.

Harding, N. E., J. M. Cleary, D. K. Cabanas, I. G. Rosen and K. S. Kang (1987). Genetic and physical analyses of a cluster of genes essential for xanthan gum biosynthesis in *Xanthomonas campestris*. J Bacteriol **169**(6): 2854-2861.

Ielpi, L., R. O. Couso and M. A. Dankert (1993). Sequential assembly and polymerization of the polyprenol-linked pentasaccharide repeating unit of the xanthan polysaccharide in *Xanthomonas campestris*. J Bacteriol **175**(9): 2490-2500.

Iguchi, M., S. Yamanaka and A. Budhioko (2000). Bacterial cellulose - a masterpiece of nature's arts. J Mater Sci **35**: 261-270.

Iliev, I., E. Radoilska, I. Ivanova and R. Enikova (2001). Biosynthesis of exopolysaccharides by two strains of *Lactobacillus bulgaricus* in whey-based media. Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet **66**(3b): 511-516.

Katzen, F., D. U. Ferreiro, C. G. Oddo, M. V. Ielmini, A. Becker, A. Pühler and L. Ielpi (1998). *Xanthomonas campestris* pv. campestris gum mutants: effects on xanthan biosynthesis and plant virulence. J Bacteriol **180**(7): 1607-1617.

Kimura, S., H. P. Chen, I. M. Saxena, R. M. Brown, Jr. and T. Itoh (2001). Localization of c-di-GMP-binding protein with the linear terminal complexes of *Acetobacter xylinum*. J Bacteriol **183**(19): 5668-5674.

Leathers, T. D. (2005). Dextran. Biopolymers for Medical and Pharmaceutical Applications. A. Steinbüchel and R. H. Marchessault. Weinheim, Wiley-VCH.

Monchois, V., M. Vignon, P. C. Escalier, B. Svensson and R. R. Russell (2000a). Involvement of Gln937 of *Streptococcus downei* GTF-I glucansucrase in transition-state stabilization. Eur J Biochem **267**(13): 4127-4136.

Monchois, V., M. Vignon and R. R. Russell (2000b). Mutagenesis of Asp-569 of glucosyltransferase I glucansucrase modulates glucan and oligosaccharide synthesis. Appl Environ Microbiol **66**(5): 1923-1927.

Monchois, V., R. M. Willemot and P. Monsan (1999). Glucansucrases: mechanism of action and structure-function relationships. FEMS Microbiol Rev **23**(2): 131-151.

Murray, B. W., S. Takayama, J. Schultz and C. H. Wong (1996). Mechanism and specificity of human alpha-1,3-fucosyltransferase V. Biochemistry **35**(34): 11183-11195.

Olsthoorn, M. M., I. M. Lopez-Lara, B. O. Petersen, K. Bock, J. Haverkamp, H. P. Spaink and J. E. Thomas-Oates (1998). Novel branched nod factor structure results from alpha-(1-->3) fucosyl transferase activity: the major lipo-chitin oligosaccharides from *Mesorhizobium loti* strain NZP2213 bear an alpha-(1-->3) fucosyl substituent on a nonterminal backbone residue. Biochemistry **37**(25): 9024-9032.

Remaud-Simeon, M., R. M. Willemot, P. Sarcabal, G. Potocki de Montalk and P. Monsan (2000). Glucansucrases: molecular engineering and oligosaccharide synthesis. J Mol Catalysis B: Enzymatic **10**(117-128).

Remminghorst, U. and B. H. A. Rehm (2006). *In Vitro* Alginate Polymerization and the Functional Role of Alg8 in Alginate Production by *Pseudomonas aeruginosa*. Appl Environ Microbiol **72**(1): 298-305.

Römling, U. (2002). Molecular biology of cellulose production in bacteria. Res Microbiol **153**(4): 205-212.

Tarbouriech, N., S. J. Charnock and G. J. Davies (2001). Three-dimensional structures of the Mn and Mg dTDP complexes of the family GT-2 glycosyltransferase SpsA: a comparison with related NDP-sugar glycosyltransferases. J Mol Biol **314**(4): 655-661.

Ünligil, U. M., S. Zhou, S. Yuwaraj, M. Sarkar, H. Schachter and J. M. Rini (2000). X-ray crystal structure of rabbit N-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily. Embo J **19**(20): 5269-5280.

van der Drift, K. M., H. P. Spaink, G. V. Bloemberg, A. A. van Brussel, B. J. Lugtenberg, J. Haverkamp and J. E. Thomas-Oates (1996). *Rhizobium leguminosarum* bv. trifolii produces lipo-chitin oligosaccharides with *nodE*-dependent highly unsaturated fatty acyl moieties. An electrospray ionization and collision-induced dissociation tandem mass spectrometric study. J Biol Chem **271**(37): 22563-22569.

Williams, W. S. and R. E. Cannon (1989). Alternative Environmental Roles for Cellulose Produced by *Acetobacter xylinum*. Appl Environ Microbiol **55**(10): 2448-2452.

Chapter I

General introduction

Bacterial alginates: from biosynthesis to applications

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Abstract

Alginate is a polysaccharide belonging to the family of linear (unbranched), non-repeating copolymers, consisting of variable amounts of *β*-D-mannuronic acid and its C5-epimer *a*-L-guluronic acid linked via *β*-1,4-glycosidic bonds. Like DNA, alginate is a negatively charged polymer, imparting material properties ranging from viscous solutions to gel-like structures in the presence of divalent cations. Bacterial alginates are synthesized by only two bacterial genera, *Pseudomonas* and *Azotobacter*, and have been extensively studied over the last 40 years. While primarily synthesized in the form of poly-mannuronic acid, alginate undergoes chemical modifications comprising acetylation and epimerization, which occur during periplasmic transfer and before final export through the outer membrane. Alginate with its unique material properties and characteristics has been increasingly considered as biomaterial for medical applications. The genetic modification of alginate producing microorganisms could enable biotechnological production of new alginates with unique, tailor-made properties, suitable for medical and industrial applications.

Introduction

Alginates were discovered in the late 19th century and have since become an important industrial product that is obtained commercially by harvesting brown seaweeds from coastal regions. However, alginates produced by these brown algae are prone to compositional variability due to seasonal and environmental changes. Up to now only the two bacterial genera *Pseudomonas* and *Azotobacter* have been described to produce alginate (Fig. 1). Both genera produce alginate as an exopolymeric polysaccharide during their vegetative growth phase, but its biological function differs between the two genera. While the biological function of alginate in brown algae appears to be as a structure-forming component, as the intercellular alginate gel matrix imparts the plants with mechanical strength and flexibility (similar to pectins in higher plants), its function in bacteria is more diverse. The Gram-negative soil bacterium *Azotobacter vinelandii* undergoes a differentiation process leading to a desiccation-resistant cyst under adverse environmental conditions. The mature cysts are surrounded by two capsule-like layers (extine and intine) each containing a high proportion of alginate in order to maintain structural integrity (Moreno et al. 1998). In *Pseudomonas aeruginosa*, one of the best characterized bacterial human pathogens, alginate seems to be an important virulence factor during the infectious process of human epithelia (Gacesa & Russell 1990). Infections of the respiratory tract with *P. aeruginosa* strains, which produce copious quantities of alginate, are the major contributing factor causing high morbidity and mortality in cystic fibrosis patients (Favre-Bonte et al. 2002; Leid et al. 2005).

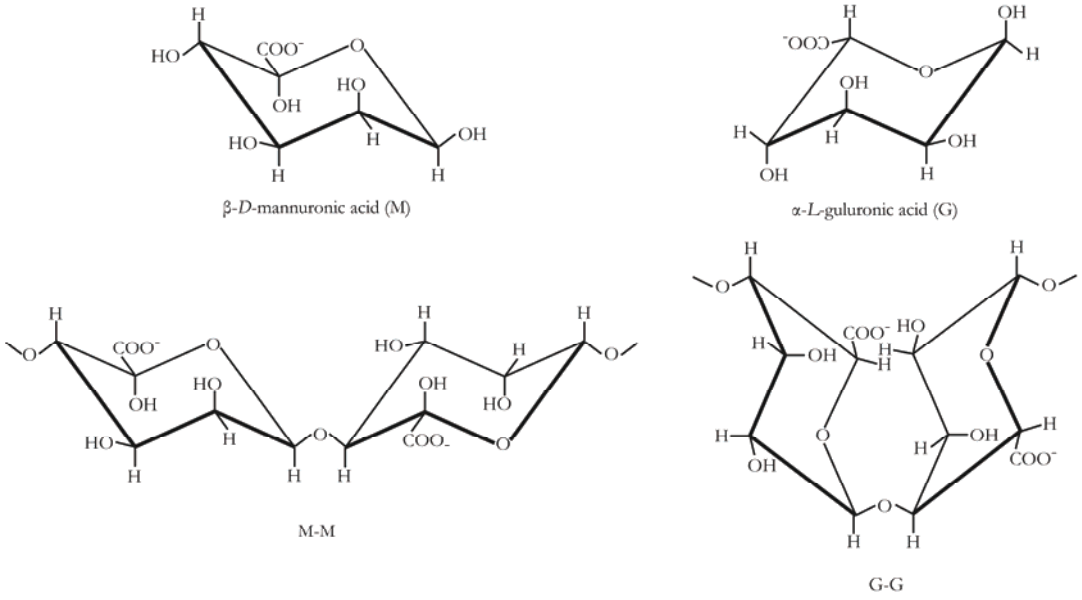

Figure 1: **Chemical structures of mannuronic (M) and guluronic (G) acid monomers and their secondary structure as blocks (MM or GG) in the alginate chain.**

The production of extensive quantities of alginate results in the development of a characteristic mucoid phenotype and is often associated with the formation of large bacterial conglomerates known as biofilms (Costerton 2001). Although biofilms are associated with numerous chronic or recurrent bacterial infections and diseases, they are ubiquitous in nature indicating a more widespread occurrence of ecological niches for mucoid forms (Donlan & Costerton 2002; Hall-Stoodley et al. 2004). Apart from the important role of alginate in biofilm formation, the polymer is often used as a biomaterial. Biomaterials play already an important role in medical applications. Polymers derived from natural sources are extensively used as biomaterials and their applications range from wound dressings, or dental implants to tissue engineering. The microencapsulation of not only drugs, but also peptides or cells, in alginate beads as controlled drug delivery systems is a rapidly growing field of applications. Nevertheless, current problems associated with purity or biocompatibility must be addressed in future to enable application in medicine, especially for naturally occurring polymers like alginate.

Genetics of alginate biosynthesis

Although regulatory genes, genotypic switch genes, and biosynthesis genes are not colocalized in bacteria, all structural genes involved in alginate biosynthesis, with the exception of *algC* (encoding phosphomannomutase), are clustered in bacteria. In *P. aeruginosa* the alginate biosynthesis cluster comprises 12 genes (*algD-algA*) under tight control of the alginate promoter upstream of *algD* (Chitnis & Ohman 1993; Shankar et al. 1995) (Fig. 2).

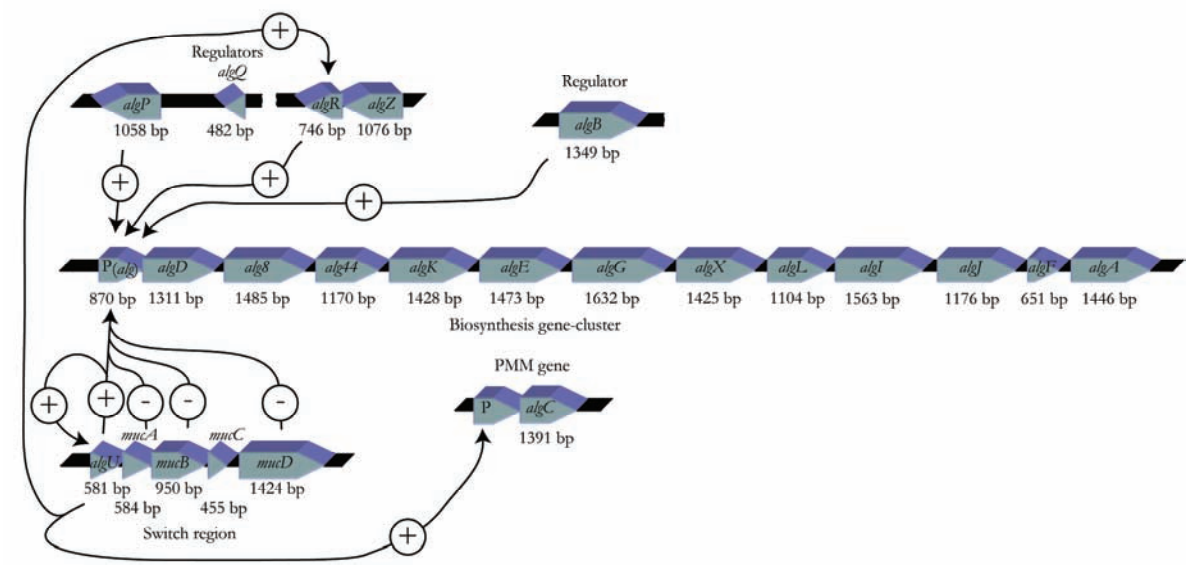

Figure 2: Simplified schematic view of the regulation network of alginate biosynthesis

This cluster includes the genes *algA* and *algD* which encode enzymes involved in precursor synthesis as well as genes encoding proteins that modify the nascent alginate chain while traversing the periplasm (*algI*, *algJ*, *algF* for acetylation, *algG* and *algL* for epimerization and degradation, respectively) and the outer membrane protein encoding gene *algE* (Schiller et al. 1993; Franklin et al. 1994; Rehm et al. 1994b; Franklin & Ohman 2002). Also located in the biosynthesis operon are the genes *algK* and *algX*, which encode periplasmic proteins putatively guiding and protecting the alginate chain against misguidance and degradation (Aarons et al. 1997; Robles-Price et al. 2004). The two genes *alg8* and *alg44* seem to be involved in the transfer of GDP-mannuronic acid across the cytoplasmic membrane. Alg8 and Alg44 were recently reported to play a role in the polymerisation process (Remminghorst & Rehm 2006a, 2006b). Although all genes share high similarities to their *A. vinelandii* counterparts, the regulation in *A. vinelandii* seems to be more complex. Two additional *algD*-independent promoters were identified as being involved in the transcription of *alg8-algJ* and *algG-algA* (Lloret et al. 1996; Vazquez et al. 1999). In general, the alginate promoter itself is regulated by various specifically and globally acting gene products (Schmitt-Andrieu & Hulen 1996) (Fig. 2). Alginate biosynthesis was found to be triggered by environmental stimuli and/or is subject to a genotypic switch involving the sigma factor AlgU and the Muc proteins (Firoved & Deretic 2003; Wu et al. 2004). Mutations in the anti-sigma factor MucA are reported to prevent its interaction with AlgU leading to transcriptional activation of the biosynthesis operon, which is associated with the change from a non-mucoid to a mucoid phenotype (Mathee et al. 1999).

Biosynthesis of alginate

Generally, the alginate biosynthesis pathway can be divided into four different stages: (i) synthesis of precursor substrate, (ii) polymerisation and cytoplasmic membrane transfer, (iii) periplasmic transfer and modification, and (iv) export through the outer membrane.

(i) Precursor synthesis

All steps involved in the conversion of central sugar metabolites into the alginate precursor GDP-mannuronic acid are well understood and have been extensively analyzed in the past. The first five steps of the precursor synthesis were initially demonstrated as enzyme activities in *Fucus gardnerii* in 1966 (Lin & Hassid 1966). The same biosynthesis steps were later found to occur also in bacteria. The biosynthesis of GDP-mannuronic acid occurs inside the bacterial cell in the cytosol. Biochemical analysis revealed that three enzymes are directly involved in the biosynthesis of GDP-mannuronic acid. In *P. aeruginosa*, a carbon source is oxidized to acetyl-CoA,

which enters into the TCA cycle, followed by the subsequent channeling of oxaloacetate via gluconeogenesis towards fructose-6-phosphate (Narbad et al. 1988) (Fig. 3). The conversion of this central sugar metabolite to mannose-6-phosphate is catalyzed by the bifunctional enzyme phosphomannose isomerase (PMI)/guanosine-diphosphomannose pyrophosphorylase (GMP), designated AlgA (PMI-GMP) (May et al. 1994). The PMI reaction pulls the fructose-6-phosphate towards alginate biosynthesis, and the resulting mannose-6-phosphate is directly converted to mannose-1-phosphate by the phosphomannomutase AlgC. This is not the only reaction catalyzed by AlgC, which also exhibits phosphoglucomutase activity and is involved in rhamnolipid and lipopolysaccharide biosynthesis (Olvera et al. 1999). The requirement of AlgC for multiple pathways might reflect its genomic localization outside the alginate biosynthesis cluster and the expression from its own promoter (Goldberg et al. 1993; Olvera et al. 1999). The GMP reaction of enzyme AlgA converts the mannose-1-phosphate into GDP-mannose with concomitant hydrolysis of GTP. The enzyme favours the reverse reaction, but the constant and very efficient removal of GDP-mannose by the guanosine-diphosphomannose dehydrogenase (GMD) AlgD drives the reaction towards GDP-mannuronate (Fig. 3). According to high intracellular levels of GDP-mannose and its utilization in various pathways, the limiting step and key kinetic control point of alginate biosynthesis is reported to be the AlgD-mediated essentially irreversible oxidation to GDP-mannuronic acid (Tatnell et al. 1994). However, recently an at least 10-times higher alginate production of *P. aeruginosa* was observed, when additional copies of Alg8 were introduced. Hence, Alg8 might be involved in another rate-limiting step of alginate production (Remminghorst & Rehm 2006b).

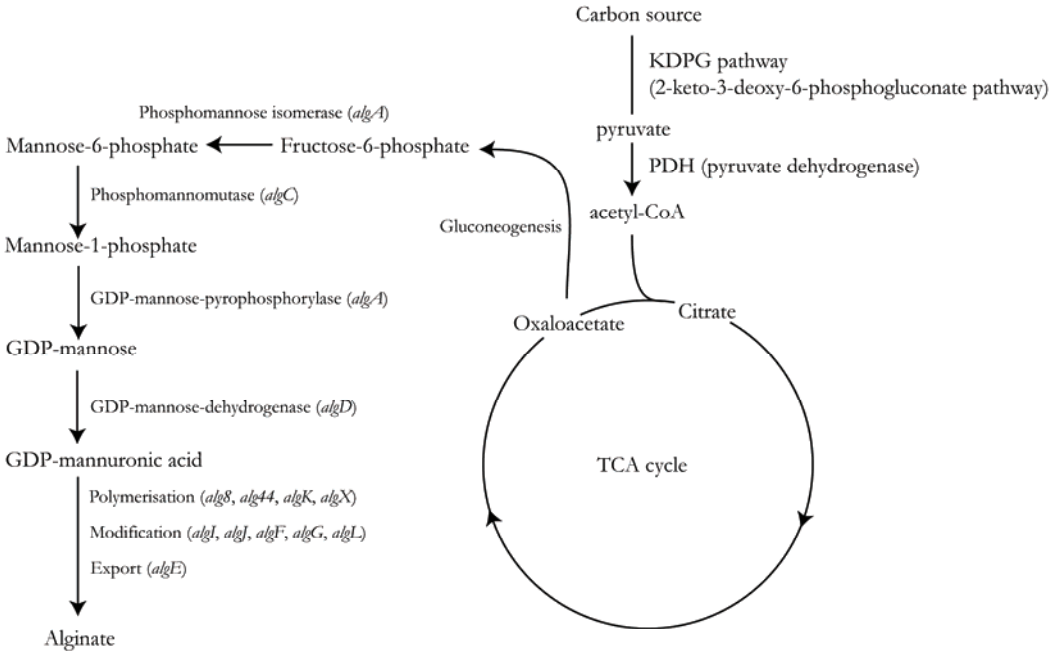

Figure 3: Proposed alginate biosynthesis pathway in *P. aeruginosa*

(ii) Polymerisation and cytoplasmic membrane transfer

The second step in alginate biosynthesis involves the transfer of the cytosolic precursor GDP-mannuronic acid across the cytoplasmic membrane and the polymerisation of the monomers to polymannuronate. Because no undecaprenol-linked intermediate has been identified in *P. aeruginosa* or *A. vinelandii*, the polymerase, presumably localized as a cytoplasmic membrane complex, is thought to catalyze alginate formation via an undecaprenol-independent mechanism (Rehm & Valla 1997). Since the alginate polymerase complex has not been purified yet, there are several possible gene products discussed as candidates for being subunits of the alginate polymerase. A recent publication suggested that Alg8 is the catalytic subunit of the alginate polymerase, because of its localization in the cytoplasmatic membrane and a proposed cytosolic loop sharing homologies to class II glycosyl-transferases (Saxena et al. 1995; Remminghorst & Rehm 2006b). Class II glycosyl-transferases catalyze the transfer of sugar residues from an activated donor substrate to an acceptor molecule, often represented by a growing carbohydrate chain. Comparison of Alg8 to functionally related enzymes (i.e. AcsAB, cellulose synthase; Chs1, chitin synthase) revealed structural similarities, suggesting polymerisation of alginate might be similar to the processive polymerisation of cellulose or chitin (for review see Saxena et al. 2001). Catalysis in these polymerisation reactions implies an inversion of the anomeric configuration at the reaction center, as the donor substrates are *a*-linked nucleotide diphospho sugars (e.g., UDP-glucose, GDP-mannuronic acid) forming a *β*-linked product (cellulose, alginate). Given that chains of polysaccharides such as cellulose or alginate adopt a 2-fold screw axis, the polymer might need to rotate while adding single molecules. However, two sugar molecules might be added in a simultaneous or sequential addition (as suggested for cellulose synthesis) to prevent chain rotation (Saxena & Brown 2000; Saxena et al. 2001). Moreover, the introduction of multiple copy numbers of Alg8 dramatically increased alginate production of *P. aeruginosa*, supporting a function as catalytic subunit of the polymerase (Remminghorst & Rehm 2006b). Unlike deletion mutants of genes *algG*, *algK* and *algX*, the *alg8* deletion mutant did not show the secretion of free uronic acids, indicating polymannuronate was not formed and subsequently degraded by the alginate lyase as suggested for the other mutants (Jain & Ohman 1998; Gimmestad et al. 2003; Jain et al. 2003; Robles-Price et al. 2004; Remminghorst & Rehm 2006b). Although Alg8 seems to catalyze the polymerisation reaction, an *in vitro* polymerisation assay revealed that only the envelope fraction (cytoplasmic membrane, periplasm, outer membrane) of *P. aeruginosa*, showed alginate polymerase activity (Remminghorst & Rehm 2006b). These recent findings and the proposed protein scaffold in the periplasm (Gimmestad et al. 2003; Jain et al. 2003; Robles-Price et al. 2004; Bakkevig et al. 2005; Jain & Ohman 2005) suggest that alginate polymerisation and export through the outer membrane might be coordinated via the formation of a multi-protein

complex involving cytoplasmic and outer membrane proteins as well as periplasmic proteins (Fig. 4).

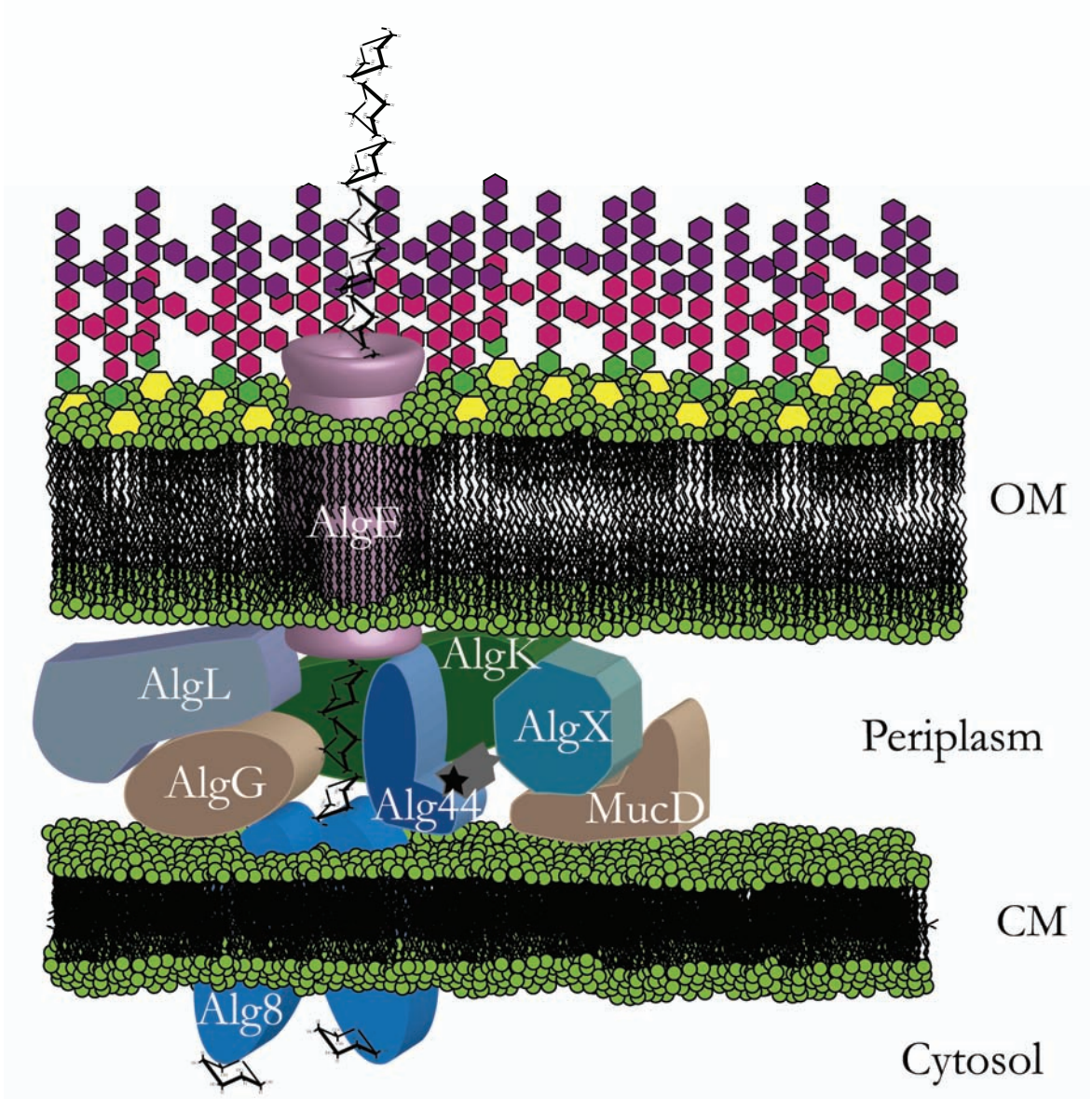

Figure 4: **Proposed model of the putative multi-enzyme complex involved in alginate polymerisation, modification and export. The star represents the effector molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP)**

The localization and function of protein Alg44 was long time unknown and many authors suggested based on its hydrophobic profile, that it is a membrane protein. Hence, interactions between Alg8 and Alg44 were suggested and both proteins were discussed to form an essential part of the polymerase complex (Salzig & Rehm 2006). Recently, it was shown that Alg44 is not associated with the membrane fraction contradicting the prediction of a transmembrane domain in Alg44 (Remminghorst & Rehm 2006a). Similarities of the C terminal part of Alg44 with MexA, a membrane bridging protein involved in the multi-drug efflux system of *P. aeruginosa* (Akama et al. 2004), support the proposed function of Alg44 as part of the periplasmic scaffold and/or

bridging Alg8 in the cytoplasmic membrane with AlgE in the outer membrane. The N terminus was reported to contain a PilZ domain (Amikam & Galperin 2006; Römling & Amikam 2006), which might be involved in binding the regulatory molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Fig. 5). Thus Alg44 might function as a regulatory membrane fusion protein (Remminghorst & Rehm 2006a) (Fig. 4).

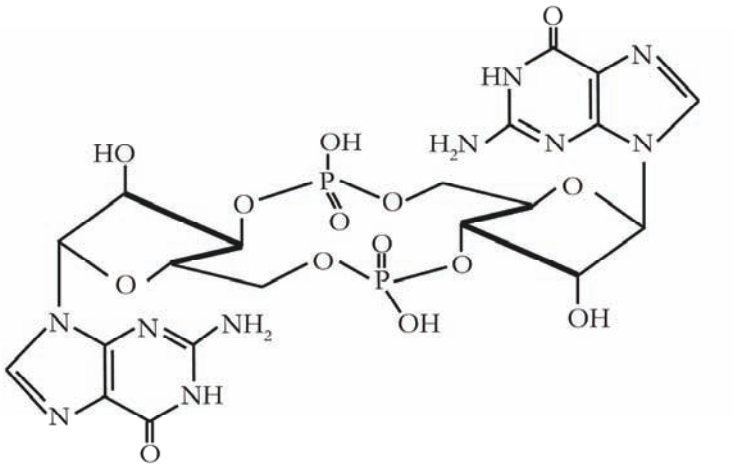

Figure 5: Chemical structure of the effector molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP)

(iii) periplasmic transfer and modification

Alginate modifying enzymes are the best analyzed proteins in the biosynthesis process, and understanding of the reaction pattern of these enzymes might enable the production of tailor-made alginates. Material properties depend predominantly on structure and composition of the alginate, so gel-forming, water-binding, and immunogenic properties of the polymer are dependent on the relative amount and sequence distribution of mannuronic and guluronic acid residues (Ertesvåg et al. 1999). Controlling the level of acetylation, epimerisation and molecular weight might open up new industrial niches for alginates. Modification of bacterial alginates occurs almost entirely in the periplasm, which implies that alginate is synthesized as polymannuronate and its modification occurs at polymer level. Additionally, the genome of *A. vinelandii* encodes a family of seven extracellular Ca^{2+}-dependend epimerases (AlgE1-7) (Ertesvåg et al. 1999; Valla et al. 2001; Bjerkan et al. 2004) and the genes encoding these extracellular epimerases are not located within the alginate biosynthesis gene cluster. Recently, six different C5-epimerase encoding genes have been identified in the genome of *Laminaria digitata* (Nyvall et al. 2003). The presence of this large gene family of epimerases suggests, even though they fulfil the same biochemical function, that these enzymes are involved in a variety of biological functions. All other genes involved in modification of the nascent alginate chain are located within the bacterial biosynthesis operon, comprising the genes encoding the acetylation-

complex (AlgI, AlgJ, AlgF), the mannuronan C5- epimerase AlgG and AlgL, the alginate lyase. In the past decade all these genes have been cloned and intensively characterized (Chitnis & Ohman 1990; Franklin & Ohman 1993; Shinabarger et al. 1993; Franklin et al. 1994; Franklin & Ohman 1996; Monday & Schiller 1996; Franklin & Ohman 2002; Gimmestad et al. 2003; Franklin et al. 2004; Albrecht & Schiller 2005; Bakkevig et al. 2005).

Transacetylation occurs at hydroxyl-groups at position C2 and/or C3 of the mannuronic acid residue, preventing these residues from being epimerized to guluronic acid and from being degraded by the alginate lyase (Franklin & Ohman 1993; Franklin et al. 1994). Thus, periplasmic acetylation indirectly controls epimerisation and degradation of the alginate polymer. By increasing the degree of acetylation, the water binding capacity of alginate is strongly enhanced, which might be particularly important for survival under dehydrating conditions (Nivens et al. 2001). Although the genes *algI*, *algJ* and *algF* are required for the addition of O-acetyl groups to the alginate polymer, acetylation itself is not required for alginate biosynthesis (Franklin & Ohman 1996). Since epimerization of the polymer was not affected in acetylation-negative mutants of *P. aeruginosa*, the acetylation complex AlgIJF does not seem to be part of the alginate protecting and aligning scaffold in the periplasm (Franklin & Ohman 2002). The proteins AlgF and AlgJ are predominantly located in the periplasm, while AlgJ is also anchored in the cytoplasmic membrane presumably by the hydrophobic part of its signal peptide. AlgI has been proposed to be a 7-helical transmembrane protein, traversing the cytoplasmic membrane and providing a yet unidentified cytosolic substrate for acetylation (Franklin & Ohman 2002).

Pseudomonas and *Azotobacter* differ with respect to epimerization of alginate. Generally, epimerases catalyze the conversion of unacetylated *β*-D-mannuronic acid residues to their C-5-epimers *a*-L-guluronic acid. But while both genera posses a periplasmic mannuronan C5-epimerase (AlgG), *Azotobacter* additionally modifies alginate by extracellular epimerases (AlgE1-7) (Rehm et al. 1996; Ertesvåg et al. 1999). The extracellular epimerases share no similarity on amino acid sequence level with their periplasmic counterparts. Interestingly, each of these seven enzymes introduced a different characteristic sequence distribution of G-residues in the polymannuronate substrate *in vitro*, which might explain the extensive variability in the alginate produced by *A. vinelandii* (Ertesvåg et al. 1999). This extensive variability might be biologically significant for the formation of the resting-stage, designated cyst, which is characteristic for the genus *Azotobacter* (Campos et al. 1996, Høidal et al. 2000). Expression of hybrid epimerase enzymes in *E. coli* demonstrated even further biotechnological potential, since the epimerization patterns of these hybrid enzymes were different from those of their parent enzymes (Bjerkan et al. 2004). The guluronic acid content of the polymer corresponds to more interaction areas with

divalent cations (especially Ca^{2+}), resulting in more rigid and gel-like polymer structures. Thus, genetic engineering or shuffling of epimerases strongly enhances the design space for alginates.

The alginate lyases, also known as alginases or alginate depolymerases, catalyze the *β*-elimination reaction leading to degradation of alginate. Generally, all lyases catalyze the same reaction, but like the epimerases, each different lyase is defined by its substrate specificity and cleavage pattern. Some lyases have preferences for glycosidic bonds between mannuronate residues, whereas others only use polyguluronate as substrate. In alginate producing bacteria, the lyase presumably functions as an editing enzyme controlling the length and molecular weight of the polymer. Alginate producing bacteria are unable to reutilize the synthesized alginate (Boyd et al. 1993).

Further proteins seem to be involved in the formation of a scaffold, which protects and aligns the nascent alginate chain while traversing the periplasm. Since deletion of *algK* and *algX* resulted in the secretion of free uronic acids presumably caused by extensive alginate lyase degradation (Jain & Ohman 1998; Robles-Price et al. 2004), the corresponding proteins were proposed to be involved in scaffold formation. The periplasmic serine protease/chaperone like protein MucD is reported to impact on regulation of alginate production (Wood & Ohman 2006). Inactivation of the proposed protease function of MucD or deletion of its gene resulted in deregulation of alginate biosynthesis in *P. aeruginosa*, phenotypically indicated by alginate production and increased temperature sensitivity. Introduction of plasmid-encoded MucD in these mutants restored regulation of alginate biosynthesis, i.e. restoring the original non-alginate producing phenotype (Wood & Ohman 2006). An interaction of AlgX with MucD was demonstrated in a recent publication, proposing that AlgX might impact on alginate formation via interaction with MucD (Gutsche et al. 2006).

Additionally, some modification enzymes are thought to be also involved in scaffold formation. For example evidence was provided that the epimerase activity of AlgG is not required for alginate production, while deletion of the full protein results in secretion of uronic acids (Gimmestad et al. 2003; Jain et al. 2003). This implied a function of AlgG in scaffold formation. Although the alginate lyase is discussed to be also part of the scaffold, it is still not fully understood whether the lyase is required for alginate biosynthesis. While in accordance with many authors, the lyase seems to be required for alginate biosynthesis by pseudomonads (Monday & Schiller 1996; Bakkevig et al. 2005; Jain & Ohman 2005), an AlgL deficient mutant of *A. vinelandii* is reported to produce reduced, but considerable amounts of alginate, implying neither structure nor function is necessary for alginate production (Trujillo-Roldan et al. 2003). In pseudomonads however, the lyase was reported to also play a role in clearing the periplasm of misguided alginate (Bakkevig et al. 2005; Jain & Ohman 2005).

(iv) export through the outer membrane

The alginate export porin AlgE was firstly described in 1990 as an outer membrane protein, highly associated with the mucoid phenotype of *P. aeruginosa* (Grabert et al. 1990; Rehm et al. 1994b). Biochemical and electrophysiological analyses of AlgE suggested a function in alginate export. In topological studies Rehm and coworkers discovered that AlgE formed an anion-selective pore through the outer membrane, and lipid bilayer experiments showed that the pore can be partially blocked by GDP-mannuronic acid (Rehm et al. 1994a). A topological three-dimensional model of AlgE has been developed by homology modeling, and according to the model, the protein is a β-barrel consisting of 18 antiparallel β-strands with 8 periplasmic 9 surface associated loops (Rehm et al. 1994a). This protein model is consistent with the proposed alginate export function, demonstrating that pore diameter allows transport of the nascent alginate chain. The protein AlgJ in *A. vinelandii* shares high similarity with AlgE, and is thought to exert the same functions as its *Pseudomonas* counterpart (Rehm 1996).

Applications of alginates

Alginate is currently used for a variety of industrial purposes and the production is hitherto exclusively based on brown seaweeds. Besides their traditional applications as food additives, alginates have been increasingly applied in the medical field particularly as dressing in surgery and wound management (Thomas 2000). Alginate is also used in controlled release of medical drugs and other chemicals. The active ingredient is entrapped in calcium alginate beads and is slowly released upon exposure to certain environmental conditions. Alginate microspheres, sometimes coated with other compounds to increase mechanical stability, have been developed and tested as oral controlled-release systems for drug delivery. Since the prices of algal alginates are low, establishment of bacterial production processes appear to be rather difficult unless high-value applications are targeted. Up to now, most bacterial alginate fermentations use *A. vinelandii* as producing organism, but even under optimized fermentation conditions production yields are quite low (around 4 g/l) compared to other microbial polymer fermentations [e.g. 30 g/l for xanthan, 23 g/l for poly(glutamic acid) or pullulan] (Richard & Margaritis 2004). It has been reported, that the molecular weight of alginate produced by *A. vinelandii* varies with respect to dissolved oxygen levels in the production process (Trujillo-Roldan et al. 2004), so new techniques or production hosts might have to be developed in order to get a commercially attractive alginate production. The past decades provided extensive insights into the molecular basis of bacterial alginate biosynthesis, and this knowledge is an important foundation for manipulation and exploitation of the microbial production process. Strategies for the production of high-quality

and/or tailor-made bacterial alginates with improved properties can be envisaged based on recent advances in alginate research. Since material properties of alginates are determined by their molecular weight, degree of acetylation, monomer composition and sequence pattern, it seems likely that all these parameters can be controlled by genetic engineering of bacterial producers. An interesting potential provided by bacterial alginates is based upon the possibility of controlling their monomer composition as well as sequential structures. Strains producing inactive mannuronan C5-epimerases enable the production of pure polymannuronate (Franklin et al. 1994). Polymannuronate, or its degradation product mannuronic acid, are known to exert anti-inflammatory efficacy (Mirshafiey et al. 2005), and could have commercial potential in applications where this is of interest. The polymannuronates could be epimerized by one particular recombinantly produced epimerase, an engineered enzyme or a combination of such enzymes, resulting in tailor-made alginates with defined properties of interest. Besides the bioengineering of alginates, enormous design space is provided by the combination of biopolymers with alginate. Recent studies indicate a promising potential of composites for future applications, particularly in the medical field (King et al. 2003; Pandey & Khuller 2004; Rehm 2005; Salzig & Rehm 2006). Alginate particles have been extensively used in medical research or transplantation techniques for immobilization of cells (Kulseng et al. 1999; Rokstad et al. 2001).

Conclusions and future perspectives

Over the last decade, the continuous development of genetic tools for manipulation of bacterial genes and genomes has generated a deeper insight into molecular mechanism of microbial alginate biosynthesis. Although the current commercial use of alginate depends almost entirely on cheap algal sources, the understanding of bacterial alginate biosynthesis will increasingly provide molecular tools for the production of tailor-made polymers. Since crucial knowledge has been gained on how the structural composition influences the material properties and on how these features can be applied in biomedical research, the tailor-made bacterial alginates might become important biomaterials in future. The growing demand for alginates adopting certain material properties will probably lead to the establishment of an adequate and efficient microbial production process. Additionally, the possibility of upgrading cheap algal alginates *in vitro* using biotechnologically produced alginate modifying enzymes will be an attractive industrial process to refine low-value alginates.

References

Aarons SJ, Sutherland IW, Chakrabarty AM, Gallagher MP (1997). A novel gene, *algK*, from the alginate biosynthesis cluster of *Pseudomonas aeruginosa*. Microbiology **143** (Pt 2): 641-652.

Akama H, Matsuura T, Kashiwagi S, Yoneyama H, Narita S, Tsukihara T, Nakagawa A, Nakae T (2004). Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. J Biol Chem **279**(25): 25939-25942.

Albrecht MT, Schiller NL (2005). Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol **187**(11): 3869-3872.

Amikam D, Galperin MY (2006). PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics **22**(1): 3-6.

Bakkevig K, Sletta H, Gimmestad M, Aune R, Ertesvåg H, Degnes K, Christensen BE, Ellingsen TE, Valla S (2005). Role of the *Pseudomonas fluorescens* Alginate Lyase (AlgL) in Clearing the Periplasm of Alginates Not Exported to the Extracellular Environment. J Bacteriol **187**(24): 8375-8384.

Bjerkan TM, Lillehov BE, Strand WI, Skjåk-Bræk G, Valla S, Ertesvåg H (2004). Construction and analyses of hybrid *Azotobacter vinelandii* mannuronan C-5 epimerases with new epimerization pattern characteristics. Biochem J **381**(Pt 3): 813-821.

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

Campos M, Martinez-Salazar JM, Lloret L, Moreno S, Nunez C, Espin G, Soberon-Chavez G (1996). Characterization of the gene coding for GDP-mannose dehydrogenase (*algD*) from *Azotobacter vinelandii*. J Bacteriol **178**(7): 1793-1799.

Chitnis CE, Ohman DE (1990). Cloning of *Pseudomonas aeruginosa algG*, which controls alginate structure. J Bacteriol **172**(6): 2894-2900.

Chitnis CE, Ohman DE (1993). Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol Microbiol **8**(3): 583-593.

Costerton JW (2001). Cystic fibrosis pathogenesis and the role of biofilms in persistent infection. Trends Microbiol **9**(2): 50-52.

Donlan RM, Costerton JW (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev **15**(2): 167-193.

Ertesvåg H, Høidal HK, Schjerven H, Svanem BI, Valla S (1999). Mannuronan C-5-epimerases and their application for *in vitro* and *in vivo* design of new alginates useful in biotechnology. Metab Eng **1**(3): 262-269.

Favre-Bonte S, Pache JC, Robert J, Blanc D, Pechere JC, van Delden C (2002). Detection of *Pseudomonas aeruginosa* cell-to-cell signals in lung tissue of cystic fibrosis patients. Microb Pathog **32**(3): 143-147.

Firoved AM, Deretic V (2003). Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. J Bacteriol **185**(3): 1071-1081.

Franklin MJ, Chitnis CE, Gacesa P, Sonesson A, White DC, Ohman DE (1994). *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. J Bacteriol **176**(7): 1821-1830.

Franklin MJ, Douthit SA, McClure MA (2004). Evidence that the *algI*/*algJ* gene cassette, required for O acetylation of *Pseudomonas aeruginosa* alginate, evolved by lateral gene transfer. J Bacteriol **186**(14): 4759-4773.

Franklin MJ, Ohman DE (1993). Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J Bacteriol **175**(16): 5057-5065.

Franklin MJ, Ohman DE (1996). Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. J Bacteriol **178**(8): 2186-2195.

Franklin MJ, Ohman DE (2002). Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. J Bacteriol **184**(11): 3000-3007.

Gacesa P, Russell NJ (1990). The structure and property of alginate. In: *Pseudomonas* Infection and alginates. London, Chapman & Hall, pp 29-49.

Gimmestad M, Sletta H, Ertesvåg H, Bakkevig K, Jain S, Suh S-J, Skjåk-Bræk G, Ellingsen TE, Ohman DE, Valla S (2003). The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J. Bacteriol. **185**: 3515-3523.

Goldberg JB, Hatano K, Pier GB (1993). Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. J. Bacteriol. **175**: 1605-1611.

Grabert E, Wingender J, Winkler UK (1990). An outer membrane protein characteristic of mucoid strains of *Pseudomonas aeruginosa*. FEMS Microbiol Lett **56**(1-2): 83-87.

Gutsche J, Remminghorst U, Rehm BHA (2006). Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie **88**(3-4): 245-251.

Hall-Stoodley L, Costerton JW, Stoodley P (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol **2**(2): 95-108.

Høidal HK, Glaerum Svanem BI, Gimmestad M, Valla S (2000). Mannuronan C-5 epimerases and cellular differentiation of *Azotobacter vinelandii*. Environ Microbiol **2**(1): 27-38.

Jain S, Franklin MJ, Ertesvåg H, Valla S, Ohman DE (2003). The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol Microbiol **47**(4): 1123-1133.

Jain S, Ohman DE (1998). Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J Bacteriol **180**(3): 634-641.

Jain S, Ohman DE (2005). Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infect Immun **73**(10): 6429-6436.

King A, Strand B, Rokstad AM, Kulseng B, Andersson A, Skjåk-Bræk G, Sandler S (2003). Improvement of the biocompatibility of alginate/poly-L-lysine/alginate microcapsules by the use of epimerized alginate as a coating. J Biomed Mater Res A **64**(3): 533-539.

Kulseng B, Skjåk-Bræk G, Ryan L, Andersson A, King A, Faxvaag A, Espevik T (1999). Transplantation of alginate microcapsules: generation of antibodies against alginates and encapsulated porcine islet-like cell clusters. Transplantation **67**(7): 978-984.

Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK (2005). The Exopolysaccharide Alginate Protects *Pseudomonas aeruginosa* Biofilm Bacteria from IFN-γ-Mediated Macrophage Killing. J Immunol **175**(11): 7512-7518.

Lin TY, Hassid WZ (1966). Pathway of algnic acid synthesis in the marine brown alga, *Fucus gardneri* Silva. J Biol Chem **241**(22): 5284-5297.

Lloret L, Barreto R, Leon R, Moreno S, Martinez-Salazar J, Espin G, Soberon-Chavez G (1996). Genetic analysis of the transcriptional arrangement of *Azotobacter vinelandii* alginate biosynthetic genes: identification of two independent promoters. Mol Microbiol **21**(3): 449-457.

Mathee K, Sternberg C, Ciofu O, Jensen P, Campbell J, Givskov M, Ohman DE, Hoiby N, Molin S, Kharazmi A (1999). Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiol. **145**: 1349-1357.

May TB, Shinabarger D, Boyd A, Chakrabarty AM (1994). Identification of amino acid residues involved in the activity of phosphomannose isomerase-guanosine 5'-diphospho-D-mannose pyrophosphorylase. A bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*. J Biol Chem **269**(7): 4872-4877.

Mirshafiey A, Matsuo H, Nakane S, Rehm BHA, Koh CS, Miyoshi S (2005). Novel immunosuppressive therapy by M2000 in experimental multiple sclerosis. Immunopharmacol Immunotoxicol **27**(2): 255-265.

Monday SR, Schiller NL (1996). Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX. J Bacteriol **178**(3): 625-632.

Moreno S, Najera R, Guzman J, Soberon-Chavez G, Espin G (1998). Role of alternative sigma factor *algU* in encystment of *Azotobacter vinelandii*. J Bacteriol **180**(10): 2766-2769.

Narbad A, Russell NJ, Gacesa P (1988). Radiolabelling patterns in alginate of *Pseudomonas aeruginosa* synthesized from specifically-labelled ^{14}C-monosaccharide precursors. Microbios **54**(220-221): 171-179.

Nivens DE, Ohman DE, Williams J, Franklin MJ (2001). Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J Bacteriol **183**(3): 1047-1057.

Nyvall P, Corre E, Boisset C, Barbeyron T, Rousvoal S, Scornet D, Kloareg B, Boyen C (2003). Characterization of mannuronan C-5-epimerase genes from the brown alga *Laminaria digitata*. Plant Physiol **133**(2): 726-735.

Olvera C, Goldberg JB, Sanchez R, Soberon-Chavez G (1999). The *Pseudomonas aeruginosa algC* gene product participates in rhamnolipid biosynthesis. FEMS Microbiol Lett **179**(1): 85-90.

Pandey R, Khuller GK (2004). Chemotherapeutic potential of alginate-chitosan microspheres as anti-tubercular drug carriers. J Antimicrob Chemother **53**(4): 635-640.

Rehm BHA (1996). The *Azotobacter vinelandii* gene *algJ* encodes an outer membrane protein presumably involved in export of alginate. Microbiology **142**: 873-880.

Rehm BHA, Boheim G, Tommassen J, Winkler UK (1994a). Overexpression of *algE* in *Escherichia coli*: subcellular localization, purification, and ion channel properties. J. Bacteriol. **176**: 5639-5647.

Rehm BHA, Ertesvåg H, Valla S (1996). A new *Azotobacter vinelandii* mannuronan C-5-epimerase gene (*algG*) is part of an *alg* gene cluster physically organized in a manner similar to that in *Pseudomonas aeruginosa*. J Bacteriol **178**(20): 5884-5889.

Rehm BHA, Grabert E, Hein J, Winkler UK (1994b). Antibody response of rabbits and cystic fibrosis patients to an alginate-specific outer membrane protein of a mucoid strain of *Pseudomonas aeruginosa*. Microb Pathog **16**(1): 43-51.

Rehm BHA, Valla S (1997). Bacterial alginates: biosynthesis and applications. Appl Microbiol Biotechnol **48**(3): 281-288.

Rehm BHA (2005). Biosynthesis and Applications of Alginates. In: Francis T (ed) Encyclopedia of Biomaterials and Biomedical Engineering, vol 1, pp 1-9.

Remminghorst U, Rehm BHA (2006a). Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. FEBS Lett **580**(16): 3883-3888.

Remminghorst U, Rehm BHA (2006b). *In vitro* Alginate Polymerisation and the Functional Role of Alg8 in Alginate Production by *Pseudomonas aeruginosa*. Appl Environ Microbiol **72**(1): 298-305.

Richard A, Margaritis A (2004). Empirical modeling of batch fermentation kinetics for poly(glutamic acid) production and other microbial biopolymers. Biotechnol Bioeng **87**(4): 501-515.

Robles-Price A, Wong TY, Sletta H, Valla S, Schiller NL (2004). AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. J Bacteriol **186**(21): 7369-7377.

Rokstad AM, Kulseng B, Strand BL, Skjåk-Bræk G, Espevik T (2001). Transplantation of alginate microcapsules with proliferating cells in mice: capsular overgrowth and survival of encapsulated cells of mice and human origin. Ann N Y Acad Sci **944**: 216-225.

Römling U, Amikam D (2006). Cyclic di-GMP as a second messenger. Curr Opin Microbiol **9**(2): 218-228.

Salzig M, Rehm BHA (2006). Microbial Production of Alginates: Self assembly and Applications. In: Rehm BHA (ed) Microbial Bionanotechnology: Biological Self-Assembly Systems and Biopolymer-Based Nanostructures. Horizon Bioscience, pp 125-152.

Saxena IM, Brown RM Jr. (2000). Cellulose synthases and related enzymes. Curr Opin Plant Biol **3**(6): 523-531.

Saxena IM, Brown RM Jr., Dandekar T (2001). Structure--function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57**(7): 1135-1148.

Saxena IM, Brown RM Jr., Fevre M, Geremia RA, Henrissat B (1995). Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. J Bacteriol **177**(6): 1419-1424.

Schiller NL, Monday SR, Boyd CM, Keen NT, Ohman DE (1993). Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (*algL*): cloning, sequencing, and expression in *Escherichia coli*. J Bacteriol **175**(15): 4780-4789.

Schmitt-Andrieu L, Hulen C (1996). Alginates of *Pseudomonas aeruginosa*: a complex regulation of the pathway of biosynthesis. C R Acad Sci III **319**(3): 153-160.

Shankar S, Ye RW, Schlictman D, Chakrabarty AM (1995). Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. Adv. Enzymol. Relat. Areas Mol. Biol. **70**: 221-255.

Shinabarger D, May TB, Boyd A, Ghosh M, Chakrabarty AM (1993). Nucleotide sequence and expression of the *Pseudomonas aeruginosa algF* gene controlling acetylation of alginate. Mol Microbiol **9**(5): 1027-1035.

Tatnell PJ, Russell NJ, Gacesa P (1994). GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. Microbiology **140** (Pt 7): 1745-1754.

Thomas S (2000). Alginate dressings in surgery and wound management--Part 1. J Wound Care **9**(2): 56-60.

Trujillo-Roldan MA, Moreno S, Espin G, Galindo E (2004). The roles of oxygen and alginate-lyase in determining the molecular weight of alginate produced by *Azotobacter vinelandii*. Appl Microbiol Biotechnol **63**(6): 742-747.

Trujillo-Roldan MA, Moreno S, Segura D, Galindo E, Espin G (2003). Alginate production by an *Azotobacter vinelandii* mutant unable to produce alginate lyase. Appl Microbiol Biotechnol **60**(6): 733-737.

Valla S, Li J, Ertesvåg H, Barbeyron T, Lindahl U (2001). Hexuronyl C5-epimerases in alginate and glycosaminoglycan biosynthesis. Biochimie **83**(8): 819-830.

Vazquez A, Moreno S, Guzman J, Alvarado A, Espin G (1999). Transcriptional organization of the *Azotobacter vinelandii algGXLVIFA* genes: characterization of *algF* mutants. Gene **232**(2): 217-222.

Wood LF, Ohman DE (2006). Independent regulation of MucD, an HtrA-like protease in *Pseudomonas aeruginosa*, and the role of its proteolytic motif in alginate gene regulation. J Bacteriol **188**(8): 3134-3137.

Wu W, Badrane H, Arora S, Baker HV, Jin S (2004). MucA-mediated coordination of type III secretion and alginate synthesis in *Pseudomonas aeruginosa*. J Bacteriol **186**(22): 7575-7585.

Chapter I

Aim and scope of this thesis

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Earlier studies of the alginate biosynthesis cluster revealed the possibility of two open reading frames downstream of *algD*, henceforth the names *alg8* and *alg44* were assigned to these ORFs. Although these two genes or their encoded proteins were investigated in those studies, the findings could neither be used for functional assignment of the proteins nor for demonstration of their requirement for alginate biosynthesis in *P. aeruginosa*. A transposon insertion into the designated ORFs of *alg8* and *alg44* impacted on alginate production as indicated by non mucoid phenotypes of the respective mutants. However, a significant decrease of enzyme activities of proteins encoded by genes further downstream in the biosynthesis operon (e.g. AlgA) suggested a polar effect of the transposon insertion on the biosynthesis cluster (see also **Chapter I** – General introduction). Although transposon-insertion studies have been extensively used in attempts to disrupt genes in various bacterial organisms, these insertions were often concomitant with downstream effects on other genes in the genomic region.

The scope of this thesis was to investigate the requirement of Alg8 and Alg44 for alginate biosynthesis in *P. aeruginosa* and to further characterise and subcellular localise these two proteins. Alg8, as the putative catalytic subunit of the alginate polymerase, should be functionally assigned and analysed at primary structure level. Knowledge about the catalytic activity could be used for enzyme inhibitor generation which could enable medical treatment of cystic fibrosis patients.

In this study, the first marker-free nonpolar isogenic knock out mutants of genes *alg8* and *alg44* were generated, respectively, and the resulting mutants were intensively studied and characterised. In **Chapter II** and **Chapter III** of this thesis, the generation of these knock outs and their complementations were studied and analysed at the molecular level. Reporter gene fusions were used to gain further understanding of the subcellular localisation of the proteins and to investigate the protein topology. **Chapter IV** explores the protein Alg8 at molecular level, investigating the site-specific replacement of single amino acids in order to resolve the current lack of knowledge about the catalytic mechanism. **Chapter V** deals with the results of the Alg8 complementation experiments and the unexpected overproducing phenotype of the complemented mutant. It investigates the impact of alginate overproduction on biofilms and cellular attachment. **Chapter VI** focuses on AlgX, another protein encoded by a gene in the alginate biosynthesis gene cluster, and its involvement in alginate production. The main results of this thesis are summarized and comprehensively discussed in **Chapter VII**.

Chapter II

In vitro alginate polymerisation and functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*

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Abstract

An enzymatic *in vitro* alginate polymerisation assay was developed using ^{14}C-labeled GDP-mannuronic acid as a substrate and subcellular fractions of alginate overproducing *Pseudomonas aeruginosa* FRD1 as a polymerase source. The highest specific alginate polymerase activity was detected in the envelope fraction, suggesting that cytoplasmic and outer membrane proteins constitute the functional alginate polymerase complex. Accordingly, no alginate polymerase activity was detected using cytoplasmic membrane or outer membrane proteins, respectively. To determine the requirement of Alg8, which has been proposed as catalytic subunit of alginate polymerase, nonpolar isogenic *alg8* knockout mutants of alginate-overproducing *P. aeruginosa* FRD1 and *P. aeruginosa* PDO300 were constructed, respectively. These mutants were deficient in alginate biosynthesis, and alginate production was restored by introducing only the *alg8* gene. Surprisingly, this resulted in significant alginate overproduction of the complemented *P. aeruginosa* Δalg8 mutants compared to nonmutated strains, suggesting that Alg8 is the bottleneck in alginate biosynthesis. ^{1}H-NMR analysis of alginate isolated from these complemented mutants showed that the degree of acetylation increased from 4.7 to 9.3 % and guluronic acid content was reduced from 38 to 19 %. Protein topology prediction indicated that Alg8 is a membrane protein. Fusion protein analysis provided evidence that Alg8 is located in the cytoplasmic membrane with a periplasmic C terminus. Subcellular fractionation suggested that the highest specific PhoA activity of Alg8-PhoA is present in the cytoplasmic membrane. A structural model of Alg8 based on the structure of SpsA from *Bacillus subtilis* was developed.

Introduction

Bacterial alginates are linear exopolysaccharides consisting of *β*-1,4-linked *β*-D-mannuronic acid and its C5 epimer *a*-L-guluronic acid (10, 15). Only the two bacterial genera *Pseudomonas* (29) and *Azotobacter* (43) are known to produce alginates. Although the polymer is primarily synthesized as polymannuronate (62), the monomer distribution is variable throughout the polymer due to epimerization of mannuronic acid residues (58, 62). Bacterial alginates can be acetylated at O2 and/or O3 positions of mannuronic acids residues (62). The best-characterized alginate-producing organism is *Pseudomonas aeruginosa*, an opportunistic human pathogen. Alginate is one important virulence factor, and the conversion of the nonmucoid to the alginate-overproducing mucoid form after infection of cystic fibrosis patients is associated with a decline of pulmonary function and survival rate (41). Alginate acts as an extracellular matrix material that allows the formation of differentiated biofilms, which restrict diffusion of clinical antibiotics and protect embedded cells against human antibacterial defence mechanisms (30, 39, 42).

Most of the genes involved in alginate biosynthesis are clustered in *P. aeruginosa* at 34 min of the bacterial chromosome (5) and are separated from other regulatory genes such as *algU* and the *muc* genes (68 min) (8, 33). The biosynthesis cluster is an operon and comprises 12 genes (*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, *algA*) under tight control of the alginate promoter upstream of *algD* (59, 60). *AlgC* is the only gene involved in alginate synthesis that is not located in the cluster, but it is also involved in lipopolysaccharide synthesis and expressed from its own promoter (17, 64). The alginate biosynthesis pathway can be divided into four different stages: (i) synthesis of precursor substrate, (ii) polymerisation and cytoplasmic membrane transfer, (iii) periplasmic modification, and (iv) export through the outer membrane. The precursor synthesis is well characterized and starts from the central metabolite fructose-6-phosphate, which is converted to GDP-mannuronic acid in four enzymatic steps by the proteins AlgA, AlgC and AlgD (45). The modifications of the polymannuronate chain are carried out by a number of periplasmic proteins. The three proteins AlgI, AlgJ and AlgF form an enzyme complex that catalyzes the O acetylation of mannuronic acid residues (12-14). AlgG is a C5-mannuronan-epimerase (11), and AlgL is an alginate lyase (38, 55). The export or secretion of the polymer chain through the outer membrane is mediated by AlgE, an alginate-specific outer membrane channel (47, 48).

The polymerisation step is still not understood. The proteins Alg8, putatively encoding a glycosyltransferase, and Alg44 are supposed to be transmembrane proteins and therefore possible subunits of the alginate polymerase (31, 35). The proteins AlgK and AlgX are periplasmic proteins, and deletion mutants showed secretion of free uronic acids presumably due to alginate

lyase activity (24, 49). Together with AlgG, these proteins are supposed to be part of a scaffold surrounding the nascent alginate chain (16, 23, 49).

In the present study we establish for the first time an enzymatic *in vitro* alginate polymerase assay enabling the subcellular localization of the respective enzyme activity. Previous studies applied transposon mutagenesis and complementation studies to investigate the role of Alg8 in alginate biosynthesis (31, 63). Here we generated a nonpolar deletion mutant of *alg8* to reveal the essential role of Alg8 in alginate polymerisation. Evidence was provided that Alg8 is the bottleneck in alginate biosynthesis. Moreover, a structural model of Alg8 was developed, and evidence for the subcellular localization of Alg8 was obtained by using translational fusions with reporter enzymes.

Materials and Methods

Bacterial Strains and Growth Conditions.

Bacterial strains, plasmids, and oligonucleotides used in the present study are listed in Table 1. *Escherichia coli* strains were grown in LB medium at 37 °C. *E. coli* S17-1 was used for conjugative transfer of *mob* site containing plasmids (61). When required, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; gentamycin, 10 μg/ml; and tetracycline, 12.5 μg/ml. *P. aeruginosa* FRD1 (40) and PDO300 (34) are used in the present study. *P. aeruginosa* strains were grown in LB or PI(A) medium (*Pseudomonas* isolation [agar] medium: 20 g peptone, 10 g K_2SO_4, 1.4 g $MgCl_2$, 0.025 g of Triclosan, and 20 ml of glycerol per liter) at 37 °C and, if required, antibiotics were added to appropriate concentrations. The antibiotic concentrations used for *P. aeruginosa* strains were as follows: gentamycin, 300 μg/ml; carbenicillin, 300 μg/ml. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation, analysis, and manipulation of DNA.

General cloning procedures were performed as described previously (51). DNA primers, deoxynucleoside triphosphate, *Taq*, and Platinum *Pfx* polymerases were purchased from Invitrogen. DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using an ABI310 automatic sequencer.

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

Strains, plasmids or oligonucleotides	Description or sequence	Source or reference
Strains		
P. aeruginosa		
FRD1	Cystic fibrosis isolate; Alg^+	(35)
FRDΔalg8Gm	Δ*alg8*::*aacC1*; Alg^-	This study
FRDΔalg8	Δ*alg8*; Alg^-	This study
PAO1	Prototrophic wild-type strain; Alg^-	(65)
PDO300	Δ*mucA22* variant of PAO1; Alg^+	(36)
PDO300Δalg8Gm	Δ*alg8*::*aacC1*; Alg^-	This study
PDO300Δalg8	Δ*alg8*; Alg^-	This study
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi*-1; *proA*, *hsdR17* (r_k^- m_k^+) *recA1*; *tra*-gene of plasmid RP4 integrated in chromosome	(34)
Plasmids		
pBBR1MCS-5	Gm^r; Broad-host-range vector; P*lac*	(43)
pBBR1MCS-5:alg8	*Hin*dIII-*Bam*HI fragment comprising gene *alg8* inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg8His	*Hin*dIII-*Bam*HI fragment encoding C terminally hexahistidine-tagged Alg8 inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg8GFP	Translational Alg8-GFP fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg8lacZ	Translational Alg8-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg8phoA	Translational Alg8-PhoA fusion, inserted into vector pBBR1MCS-5	This study
pEX100T	Ap^r; Cb^r, gene replacement vector containing *sacB* gene for counterselection	(41)
pEX100TΔalg8Gm	Ap^r, Cb^r, Gm^r; vector pEX100T with *Sma*I inserted *alg8* deletion construct	This study
pPS856	Ap^r; Gm^r; source of 1,100-bp *Bam*HI-fragment comprising *aacC1* gene flanked by FRT signal sequences	(41)
pPFLP2	Ap^r; Cb^r; broad-host-range vector encoding Flp recombinase	(41)
pPHO7	Ap^r; *phoA* without signal sequence	(44)
pZsGreen	GFP translational fusion vector	BD Clonetech
pJE608	LacZ lacking the first 8 amino acids with promoter P*tac* in pMMB67EH	(45)
Oligonucleotides		
alg81N-Ec5	CGCAGGATATCGGAAACTTACAAACGTGGCCTC	
alg81N-Ba	TAGAGGATCCGGTTCATCTTCTCCCACAGAG	
alg82C-Ba	TGATGGATCCGTTCACCATGCTGGTGCTGTTC	
alg82C-Ec5	CCAGGATATCTCATACGATGGTCAGCAGCAC	
alg8up	AAGAACCTCTTTATCGCCCTCGGAC	
alg8down	TCACGGATCCCCAGGTAGGAGGTGATCAGGTAG	
alg8N(HiSDNd)	CCGGGAAGCTTGAGGAGCACAGCCATATGGAACTGATGATGGAAACT TACAAA	
alg8C(Ba)	CCCGGATCCTCATACGATGGTCAGCAGCACG	
alg8C(HisBa)	CTTGGATCCTCAATGGTGATGGTGATGGTGTACGATGGTCAGCAGCAC GGCGAC	
alg8C(Δstop)	CCCGGATCCCATACGATGGTCAGCAGCACG	

Enzymatic synthesis and purification of GDP-mannuronic acid.

GDP-mannose dehydrogenase (GMD) was partially purified from *P. aeruginosa* 8822 (7) as previously described by Roychoudhury et al. (50). GMD activity was measured according to Preiss (44) by monitoring reduction of NAD^+ to NADH at a wavelength of 340 nm. A specific activity of 570 mU/mg protein was obtained, and the substrate GDP-mannose was completely oxidized to GDP-mannuronic acid. One unit of GMD activity corresponds to the oxidation of 1 μmol GDP-mannose to GDP-mannuronic acid per min. Proteins were either removed by ultrafiltration (cutoff = 10 kDa) or, when ^{14}C-GDP-mannuronic acid was produced, by phenol-chloroform (1:1) extraction. GDP-mannuronic acid containing fractions were subjected to anion-exchange chromatography (MonoQ). GDP-mannuronic acid containing fractions were detected by uronic acid analysis and subjected to gel filtration chromatography using a Sephadex G15 column.

^{14}C-GDP-mannuronic acid and GDP-mannuronic acid were separated by thin-layer chromatography (TLC) with PEI-cellulose (Schleicher & Schuell) and the solvents 0.2 M LiCl, 1.0 M LiCl and 1.6 M LiCl. The ^{14}C-GDP-mannuronic acid was detected by autoradiography and use of a TLC scanner (Berthold LB 2760). As standards, purified GDP-mannuronic acid and GDP-mannose (Sigma-Aldrich, St. Louis, MO) were used and detected using 0.002% (wt/vol) fluorescein in methanol for staining.

Enzymatic *in vitro* alginate synthesis.

Crude extracts and cytoplasmic membrane (CM), outer membrane (OM), and envelope fractions were used as a source for alginate polymerase activity. The CM was obtained by sucrose gradient ultracentrifugation as described previously (48). All other fractions including the OM were obtained as described below. The contamination of CM with OM was estimated from its 2-keto-3-desoxyoctonate (KDO) content and was given as a percentage of the total amount of KDO present in the CM and OM (48). The contamination was determined to be about 10% as has been previously published (48).

Protein fractions contained 50 mM Tris-HCl, (pH8.0), 0.5 mM phenylmethylsulfonyl fluoride, 0.1% (vol/vol) Triton-X100 and 2 mM dithiothreitol. The *in vitro* synthesis reaction contained 143 pmol ^{14}C-GDP-mannuronic acid (286.1 mCi/mmol), 857 pmol of GDP-mannuronic acid, 50 mM Tris-HCl (pH 8.0), 10 μM $MgCl_2$, 70 μg of alginate oligomers (n = 3 to 6), and 2.5 mg of protein sample (polymerase source) in a total volume of 260 μl. The alginate oligomers were obtained by acid hydrolysis as previously described (46). As a negative control, inactive enzyme preparation was used. Inactive enzyme was obtained by heat treatment applying 100 ºC for 10 min. The synthesis reaction was started by the addition of protein sample

(polymerase source) and conducted at 37 ºC for 20 min. The reaction mixture (50 µl) was loaded onto anion-exchange filter (DE81; Whatman), and the filter was subsequently washed with 0.3 M NaCl (GDP-mannuronic acid was tested to be eluted from the filter using 0.3 M NaCl), water, and then ethanol. A total of 5 ml of scintillation cocktail [0.5% (wt/vol) 2,5-diphenyloxazol; 0.02% (wt/vol) 2,2-*p*-phenylen-bis-(5-phenyloxazol)] was added, and counts per min (cpm) were measured. The specific alginate polymerase activity is given as follows: Δcpm $[(a_{20}-a_0) - (i_{20}-i_0)]$/mg protein x min (whereas a is active enzyme; I is inactive enzyme, and 0 and 20 refer to t = 0 min and t = 20 min, respectively). In this reaction mixture, Δ100 cpm corresponded to 0.215 pg of alginate and a specific alginate polymerase activity of 1.15 µU/mg protein. One unit corresponds to the conversion of 1 µmol of GDP-mannuronic acid into alginate per min.

Construction and confirmation of *alg8* deletion mutants.

Two regions of the *alg8* gene were amplified using *Taq* polymerase and primers alg81N-Ec5, alg81N-Ba, alg82C-Ba, and alg82C-Ec5. Region alg8N (431 bp) comprised bases 15 to 445 and region alg8C (403 bp) comprised bases 1092 to 1494 relative to the designated *alg8* coding region (31), respectively. Both PCR products were hydrolysed by using *Bam*HI and inserted into vector pGEM-T_{Easy} (Promega). Vector pPS856 (20) was hydrolysed with *Bam*HI, releasing an about 1,100-bp fragment containing the *aacC1* gene (encoding gentamycin acetyltransferase) flanked by two *FRT* (Flp recombinase target) sites. The 1,100-bp *Bam*HI fragment (*aacC1* gene) was inserted into the *Bam*HI site of plasmid pGEM-T_{Easy}:Δalg8NC, resulting in plasmid pGEM-T_{Easy}:Δalg8Gm. The 1,949-bp Δalg8Gm comprising DNA fragment was amplified by *Pfx* polymerase using primers alg81N-Ec5 and alg82C-Ec5, and the corresponding PCR product was inserted into *Sma*I site of vector pEX100T (20), resulting in plasmid pEX100TΔalg8Gm.

E. coli S17-1 was used as donor for transfer of plasmid pEX100TΔalg8Gm into *P. aeruginosa* strains, and transconjugants were selected on mineral salt medium (56) containing 300 µg of gentamycin/ml and 5 % (wt/vol) sucrose. Cells growing on this selective medium should have emerged from double-crossover events. Gene replacement was confirmed after subculture of cells on PIA medium containing 300 µg of gentamycin/ml and using PCR with primers alg8up and alg8down.

E. coli S17-1 was used to transfer the Flp recombinase encoding vector pFLP2 (20) into *P. aeruginosa* Δalg8Gm strains and after 24 h of cultivation on PIA medium containing 5 % (wt/vol) sucrose, gentamycin- and carbenicillin-sensitive cells were analyzed by PCR for loss of the gentamycin-resistant cassette.

Complementation of isogenic *alg8* deletion mutants.

For complementation of *alg8* deletion mutants, the *alg8* gene of *P. aeruginosa* PAO1 was amplified by PCR with the primers alg8N(HiSDNd) and alg8C(Ba). The PCR product was hydrolysed with *Hin*dIII and *Bam*HI and was inserted into *Hin*dIII and *Bam*HI sites of broad-host-range vector pBBR1MCS-5 (28), resulting in plasmid pBBR1MCS-5:alg8. In addition, the 3'-end primer alg8C(HisBa) was used to generate an *alg8* gene encoding a C-terminally hexahistidine tagged Alg8, which was inserted into vector pBBR1MCS-5 as described above (Table 1). All inserts cloned into the multiple cloning site of vector pBBR1MCS-5 are under control of the *lac* promoter.

Subcellular localization using PhoA, LacZ and GFP fusions.

The 3'-end of the *alg8* gene was amplified by PCR using *Pfx* polymerase and primers alg82C-Ba and alg8C(Δstop). The corresponding 422-bp PCR-fragment was inserted into *Sma*I sites of pBluescriptKS(-) (Stratagene), resulting in plasmid pKS$^-$:alg8(Δstop). After *Bgl*II and *Sac*II hydrolysis, the resulting 203-bp fragment was used to replace the original 3'-end of the *alg8* gene in plasmid pBBR1MCS-5:alg8, resulting in plasmid pBBR1MCS-5:alg8(Δstop). *Xba*I-*Bam*HI fragments of vectors pPHO7 (19), pJE608 (9) and pZsGreen (BD Biosciences Clontech) were inserted into *Xba*I-*Bam*HI-restricted pBBR1MCS-5:alg8(Δstop) to create translational PhoA, LacZ, and green fluorescent protein (GFP) fusions, respectively.

Subcellular fractionation.

An overnight culture of the respective *P. aeruginosa* strain in LB medium was diluted 1:50 in the same medium and grown for 4 h or until an optical cell density at 600 nm of 0.5 to 0.6 was reached. The cultures were harvested, and cell sediments were suspended and washed twice in 10 mM HEPES buffer (pH 7.4). The cells were resuspended in 1 ml HEPES buffer (pH 7.4) and sonicated at 30 % intensity for eight cycles of 15 s sonication, followed by 20 s of cooling down. Cellular debris and unlysed cells were sedimented by centrifugation (1 h at 5,000 x *g*). Then, 800 µl of the supernatant was centrifuged at 100,000 x *g* for 2 h. The supernatant (soluble fraction) was transferred into a clean tube, and the sediments were resuspended in 800 µl of 10 mM HEPES buffer (pH 7.4) and centrifuged under the same conditions. The supernatant was again transferred into a clean tube (wash fraction), the sediment (envelope fraction) was redissolved in 800 µl of 10 mM HEPES buffer (pH 7.4) containing 0.7 % (wt/vol) *N*-lauroylsarcosine, and selective solubilization of the cytoplasmic membrane was achieved by incubation on a horizontal shaker for 1.5 h at 37 °C. The mixtures were centrifuged for 2 h at 100,000 x *g*, and the supernatant was transferred into a clean tube (solubilized cytoplasmic

membrane). This solubilization step was repeated, and the resulting membrane sediment (outer membrane) was resuspended in 800 µl of 10 mM HEPES buffer (pH 7.4).

Alkaline phosphatase / β-galactosidase activity assays.

Alkaline phosphatase and β-galactosidase enzymatic assays were performed according to the methods of Miller (36) and Manoil (32), respectively. Cells of *P. aeruginosa* were grown overnight in LB with the appropriate antibiotic, and the cultures were diluted 1:50 in LB medium. The cells were allowed to grow until the cultures reached an optical cell density at 600 nm of 0.4 to 0.6. The enzyme assays were performed in 1 ml of these cultures, and 1 ml was used for freeze-drying to determine the cellular dry weight. PhoA activity was determined by the rate of *p*-nitrophenylphosphate hydrolysis, taking the amount of enzyme hydrolyzing 1 µmol of substrate per 1 min at 37 °C as a unit of enzymatic activity. Results are given as average values of at least four independent experiments.

Alginate production assays.

A total of 2 ml of bacterial overnight cultures was harvested at 4 °C and washed twice with saline. Then, 200 µl of cell suspension was plated onto PIA medium and incubated 72 h at 37 °C. Cells of two agar plates were scraped off by using a sterile spatula and washed twice with 40 ml of saline. When viscosity of the solution was too high for separation of cells (complemented mutants), saline was added to a total volume of 300 ml to allow sedimentation of cells during centrifugation. Cellular sediments were freeze-dried, and the final weight was determined. Alginate supernatants were precipitated with 1 vol of ice-cold isopropanol, and alginate was harvested and freeze-dried. For further purification, the precipitated alginate was redissolved in 0.05 M Tris-HCl, 10 mM $MgCl_2$ (pH 7.4) to a final concentration of 0.5 % (wt/vol), followed by incubated with 15 µg of DNase I/ml and 15 µg of RNase A/ml at 37 °C for 6 h. Pronase E was added to a final concentration of 20 µg/ml, and this solution was incubated for further 18 h at 37 °C. Alginate solutions were dialyzed against 5 litres of ultrapure H_2O for 48 h. Alginate was precipitated with 1 volume of ice-cold isopropanol and freeze-dried for quantification and uronic acid analysis.

Uronic acid assays.

Alginate concentrations were assayed by a modification of the Blumenkrantz and Asboe-Hansen protocol (3), using purified *P. aeruginosa* PDO300 alginate (100 % [wt/wt] uronic acid content) as a standard. Briefly, alginate samples were dissolved in 200 µl of ultrapure H_2O at concentrations between 0.25 and 0.05 mg/ml. The sample was mixed with 1.2 ml tetraborate solution (0.0125 M

disodium tetraborate in concentrated sulphuric acid) and incubated on ice for 10 min. The mixtures were incubated at 100 °C for 5 min and then cooled down on ice for further 5 min. Then, 20 µl of *m*-hydroxybiphenyl reagent (0.15 % *m*-hydroxybiphenyl in 0.125 M NaOH) was added, and the reactions were mixed for 1 min. For each sample or dilution a negative control was assayed by using 0.125 M NaOH instead of the hydroxybiphenyl reagent. Uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

^{1}H-NMR analysis of alginates.

The alginate samples were deacetylated and partially degraded by mild, acid hydrolysis in order to reduce the viscosity of the solutions. Alginate samples were analyzed by high-field ^{1}H-nuclear magnetic resonance (NMR) spectroscopy at 90 °C by using a Bruker AM-300 (300-MHz) spectrometer. 3-(Trimethylsilyl)propanesulfonate was used as an internal standard in the samples. Prior to the NMR spectroscopy, the samples were desalted on Bio-Gel P-4 (Bio-Rad), freeze-dried, and dissolved in D_2O. The removal of salt resulted in a better signal-to-noise ratio. The composition, given as molar fraction of the monomers G (F_G) and M (F_M), and the dyads (F_{GG}, F_{GM}, and F_{MM}) were determined from the spectra as described by Grasdalen et al. (18). In this procedure, the area under each peak, which is proportional to the amount of residues giving rise to the signal, is used to calculate the above parameters.

Results

Enzymatic *in vitro* synthesis of alginate.

An enzymatic *in vitro* polymerisation assay was established using ^{14}C-labeled GDP-mannuronic acid as substrate, alginate oligomers as primer, and subcellular fractions of alginate-overproducing *P. aeruginosa* FRD1 as polymerase source. ^{14}C-GDP-mannuronic acid is commercially not available and was enzymatically synthesized by using partially purified GDP-mannose dehydrogenase and GDP-mannose as substrate. Complete oxidation of ^{14}C-GDP-mannose to ^{14}C-GDP-mannuronic acid was monitored by TLC in combination with autoradiography and densitometry. Complete oxidation of GDP-mannose to GDP-mannuronic acid was monitored by TLC and uronic acid specific staining (data not shown). Anion-exchange filters were applied to selectively bind polymerized alginate. The binding of GDP-mannuronic acid to the filter was analyzed, revealing that 0.3 M NaCl is the lowest NaCl concentration completely eluting GDP-mannuronic acid from the filter (data not shown). Alginate and alginate oligomers (n=3 to 6) did not elute after a wash with 0.3 M NaCl, enabling selective binding of polymerized GDP-mannuronic acid, which was detected by scintillation analysis. To subcellularly localize the alginate polymerase, various

subcellular fractions were analyzed with respect to alginate polymerase activity (Table 2). The highest specific alginate polymerase activity was detected in the envelope fraction, suggesting that cytoplasmic and outer membrane proteins together constitute the functional alginate polymerase complex. Accordingly, no alginate polymerase activity was detected by using cytoplasmic membrane or outer membrane proteins, respectively. The omission of the detergent Triton-X100 in the envelope fraction caused a decrease in specific alginate polymerase activity (Table 2).

Table 2: *In vitro* alginate synthesis using subcellular fractions of *P. aeruginosa* FRD1 as polymerase source.[a]

Subcellular fraction	cpm/reaction mixture		alginate polymerase sp act (μU/mg of protein)	*In vitro* synthesized alginate (pg)
	No.	(%)		
Crude extract	161	3.8	0.7	0.36
Cytoplasmic membrane	ND	ND	ND	ND
Outer membrane	ND	ND	ND	ND
Envelope	3,037	100	13.2	6.5
Envelope[b]	582	19	2.5	1.25

[a] All experiments were performed in triplicates. Average values are given. The standard deviation was <30%. ND, not detectable; [b] Triton-X100 was omitted.

Construction of an isogenic knock out mutant of *alg8*.

To investigate the requirement of Alg8 in alginate biosynthesis, marker-free *alg8* deletion mutants of alginate-overproducing strains *P. aeruginosa* PDO300 and *P. aeruginosa* FRD1 were generated, respectively (Figure 1). Both mutant strains showed a nonmucoid phenotype on agar plates, suggesting that Alg8 is required for alginate synthesis.

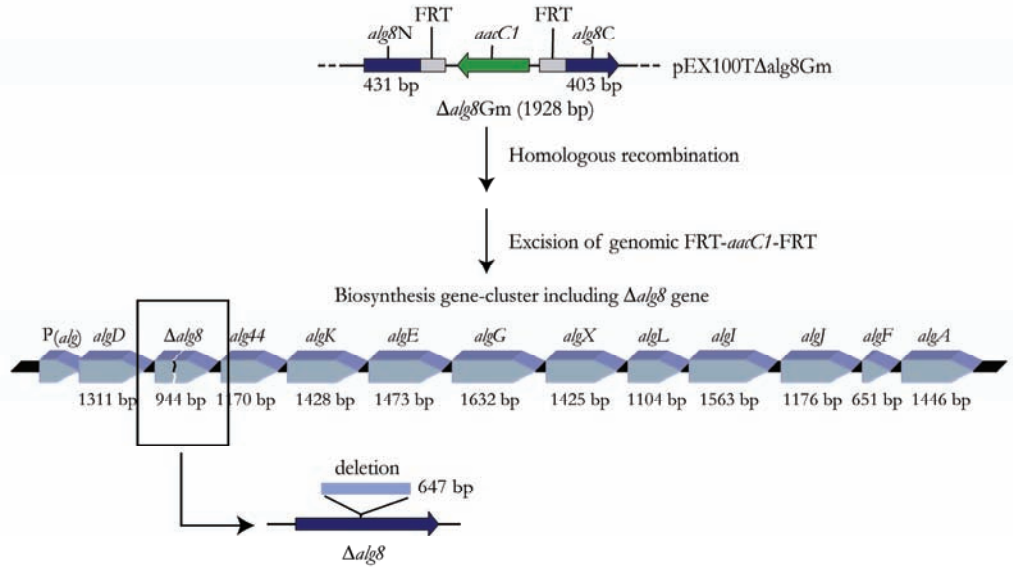

Figure 1: **Schematic view of *alg8* knockout construct of plasmid pEX100TΔalg8Gm used for homologous recombination and the alginate biosynthesis operon after replacement of native *alg8* gene with Δ*alg8*.**

Recent publications suggested that the nonmucoid phenotype of *alg* deletion mutants (*algK*, *algX*, and *algG*) was associated with the secretion of uronic acid oligomers generated by degradation of the alginate polymer by the alginate lyase AlgL (23, 49). To investigate whether the loss of mucoidy of the *alg8* mutants was associated with production of uronic acid oligomers, the respective supernatants were dialysed and/or ultrafiltrated. No free uronic acids (< 5,000 Da) were detected, suggesting that the alginate-deficient phenotype of the mutants was due to a loss of alginate formation.

Alg8 plays a key role in alginate production.

To verify that the deletion had no downstream effects within the biosynthesis operon, a plasmid containing only the ORF of *alg8* (pBBR1MCS-5:alg8) was used to restore the mucoid phenotype. Interestingly, the mucoid phenotype was not only restored, but alginate production was at least 20-fold increased compared to *P. aeruginosa* PDO300 (Table 3). Plasmid pBBR1MCS-5:alg8 mediated a 15-fold increased alginate production in *P. aeruginosa* PDO300, suggesting that Alg8 is the bottleneck in alginate production. A twofold increase of alginate production was observed when *P. aeruginosa* PDO300 (pBBR1MCS-5) was grown in the presence of gentamycin compared to the absence of gentamycin. The alginate overproduction of *alg8* mutants harbouring plasmid pBBR1MCS-5:alg8 was associated with a decrease of cellular dry mass to 54 % (wt/wt) ± 10 % (wt/wt) compared to cellular dry mass production of strain *P. aeruginosa* PDO300. A similar effect was found in *P. aeruginosa* PDO300 harbouring plasmid pBBR1MCS-5:alg8 (Table 3).

Table 3: Alginate and cellular dry mass production by different *P. aeruginosa* strains harbouring various plasmids.

Strain	Mean ± SD	
	Alginate production (g/g CDM)	Cellular dry mass (CDM [g])
PAO1	ND[a]	0.227 ± 0.021
PAO1 (pBBR1MCS-5)	ND	0.223 ± 0.029
PAO1 (pBBR1MCS-5:alg8)	ND	0.202 ± 0.007
PDO300	0.091 ± 0.028	0.103 ± 0.006
PDO300 (pBBR1MCS-5)	0.192 ± 0.004	0.115 ± 0.012
PDO300 (pBBR1MCS-5:alg8)	1.314 ± 0.045	0.076 ± 0.011
PDO300Δalg8	ND	0.101 ± 0.003
PDO300Δalg8 (pBBR1MCS-5)	ND	0.091 ± 0.002
PDO300Δalg8 (pBBR1MCS-5:alg8)	2.039 ± 0.613	0.057 ± 0.002
PDO300Δalg8 (pBBR1MCS-5:alg8His)	2.770 ± 0.545	0.046 ± 0.008
PDO300Δalg8 (pBBR1MCS-5:alg8GFP)	3.092 ± 0.241	0.053 ± 0.007
PDO300Δalg8 (pBBR1MCS-5:alg8phoA)	2.852 ± 0.387	0.046 ± 0.007
PDO300Δalg8 (pBBR1MCS-5:alg8lacZ)	2.263 ± 0.192	0.056 ± 0.011

[a] ND, not detectable

Further complementation and production analyses with mutant *P. aeruginosa* PDO300Δalg8 harbouring plasmids that encode C-terminally tagged Alg8 proteins indicated that the polymer production yield was not affected by translational fusions of Alg8. The translational hexahistidine, PhoA, GFP, and LacZ fusions mediated an at least 20-fold-increased alginate production compared to *P. aeruginosa* PDO300 (Table 3).

Transfer of plasmid pBBR1MCS-5:alg8 into *alg8* mutant of *P. aeruginosa* FRD1 restored alginate production, but a free plasmid was not detectable. Interestingly, PCR analysis and DNA sequencing confirmed that the intact *alg8* gene was inserted into the genome (data not shown).

Alg8 impacts on alginate composition.

Differences in alginate solubility and viscosity of alginates from *P. aeruginosa* PDO300 and the respective complemented *alg8* mutants indicated that polymer composition might be different. ^{1}H-NMR analysis of alginates isolated from *P. aeruginosa* PDO300 and *P. aeruginosa* PDO300Δalg8 (pBBR1MCS 5:alg8) revealed that, due to additional *alg8* gene copies, the degree of acetylation increased from 4.7 to 9.3 %, whereas the guluronic acid content of the polymer decreased from 38 to 19 %, and the frequency of the mannuronic acid doublet (F_{MM}) increased from 24 to 62 % (data not shown).

Construction and analysis of translational fusion proteins of Alg8.

Alg8 is supposed to be a membrane protein that putatively encodes a glycosyltransferase linking the cytosolic precursor synthesis to polymer formation (45). Topological analyses of Alg8 using the SMART database (57) and the topology prediction tool TMHMM2 (37) suggested a signal sequence at the N terminus (1 to 32 amino acids) and four transmembrane helices (Fig. 2). To analyse the topology and subcellular localization of Alg8, C-terminal translational fusions of Alg8 to the reporter proteins LacZ, PhoA, and GFP were constructed. All Alg8 fusions were functional and restored alginate production in *P. aeruginosa* PDO300Δalg8 (Table 3). Reporter protein assays revealed a specific alkaline phosphatase activity (PhoA Units) of 7.56 U/mg cellular dry weight and a β-galactosidase activity of 0.48 U/mg cellular dry weight. Alg8 fused to GFP did not enable localization of GFP foci using fluorescence microscopy. These data suggested a periplasmic localization of the C terminus. A recent publication reported an improved topology prediction algorithm using HMM (hidden Markov model) and experimentally verified localization of the C terminus (2). Thus, the HMM-based topology tool Phobius (http://phobius.cgb.ki.se) was used to further analyse the membrane topology using constrained prediction with a periplasmic C terminus (25). These results strongly support the model of a membrane protein containing a N-terminal signal sequence and 4 transmembrane helices (Fig. 2).

Cell fractionation experiments were performed using *P. aeruginosa* PDO300Δalg8 (pBBR1MCS-5:alg8phoA) and the envelope fraction, as well as the solubilized cytoplasmic membrane fraction, showed the highest specific alkaline phosphatase activity (PhoA Units) of 13.9 ± 0.2 U/mg of protein and 18.4 ±0.3 U/mg of protein, respectively. The cytoplasmic membrane proteins were selectively enriched by solubilization of the membrane with 0.7 % (wt/vol) *N*-lauroylsarcosine, which also solubilized the Alg8-PhoA fusion protein. The cytosolic fraction showed a PhoA activity of 9.7 ± 0.1 U/mg, and the outer membrane fraction showed an activity of 9.9 ±0.3 U/mg.

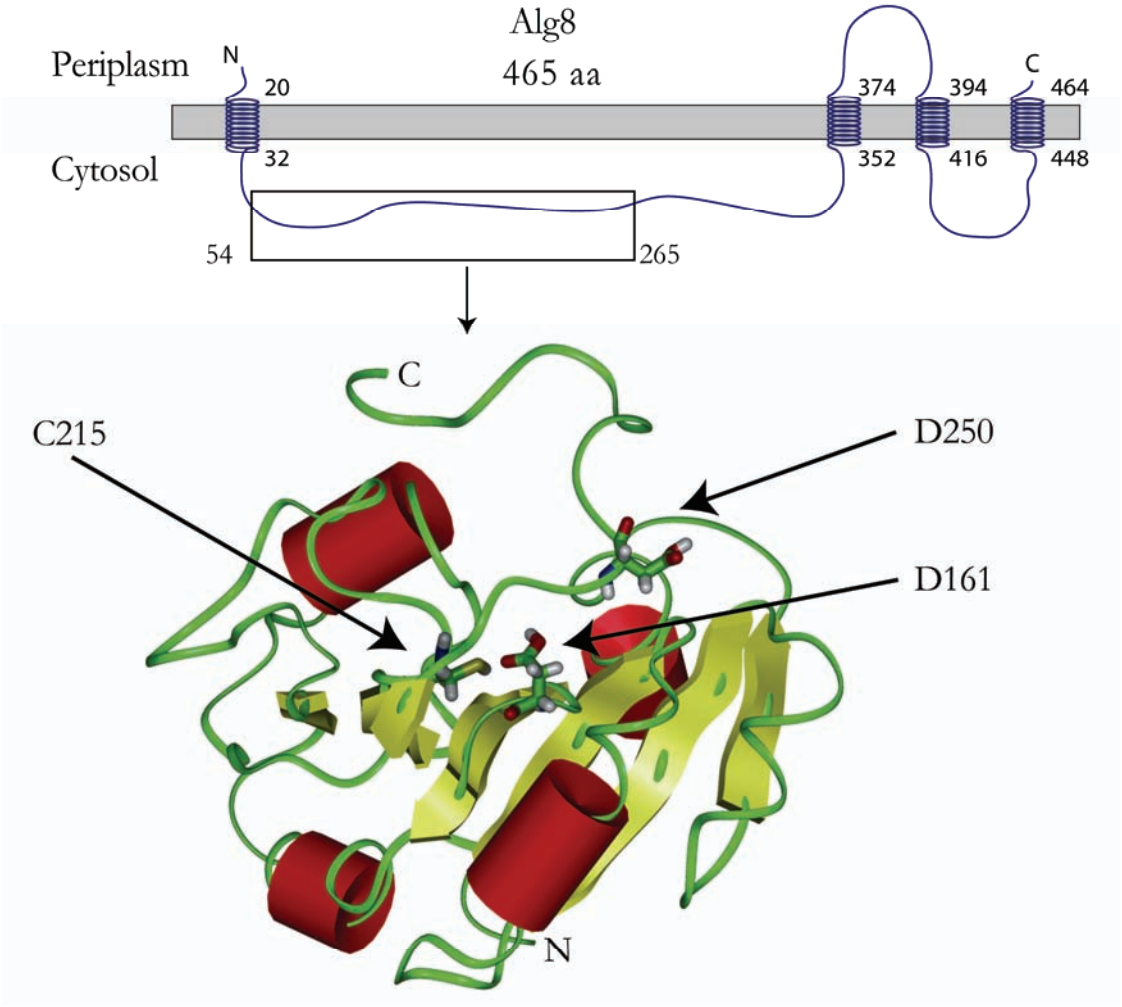

Figure 2: **Predicted membrane topology of Alg8 based on different HMM-based algorithms (Phobius, SMART, and TMHMM2) of the processed Alg8. Numbers represent the location of the amino acids in the processed form starting with first N-terminal amino acid after the predicted signal peptide cleavage site with number 1. The threading model was developed based on the SAM-T02 alignment of Alg8 with SpsA (1qg8). Cylinders represent α-helical structures. Big arrows represent β-strands. The putative catalytic residues are given as stick side chains and indicated by arrows.**

N, N terminus of the structural Alg8 model; C, C terminus of the structural Alg8 model.

Development of a threading model of Alg8.

Hydrophobic cluster analysis showed that Alg8 shares significant homologies with β-glycosyltransferases of class II (54). A SMART database search also suggested that Alg8 contains the β-glycosyltransferase domain, although the result was less significant with respect to the required threshold according to the HMM model. Submission of the Alg8 sequence to algorithms that search structural databases (SAM-T02 (26), 3D-PSSM (27)) showed 19.6 % similarity of Alg8 to the spore coat polysaccharide biosynthesis protein SpsA (1qg8a) from *Bacillus subtilis*, which represents a nucleotide-diphospho-sugar transferase (4). A threading model of Alg8 was developed with the aid of the SpsA structure as previously described (21). Inspection of the protein model showed that homologues of amino acid residues presumably forming a catalytic triad in SpsA are also adjacent to the core structure in the Alg8 model. The SpsA structure exhibits several amino acid residues serving as potential base functions that might be involved in catalytic function: such as C160 and D191. The homologous amino acid residues C215 and D250 are supposed to be candidates for catalytic residues of Alg8. Another aspartic acid residue is located in a conserved DXD motif that can be found in many glycosyltransferases (53). This motif can be found twice in Alg8 at positions 150 to 152 and 159 to 161. In SpsA the DXD motif is suggested to be reduced to a single aspartic acid residue (52) that corresponds to D161 in the Alg8 model.

Discussion

In this study, we describe the establishment of an enzymatic *in vitro* alginate synthesis assay using ^{14}C-GDP-mannuronic acid as activated alginate precursor. An efficient method for enzymatic oxidation of GDP-mannose and purification of GDP-mannuronic acid was developed. For the first time alginate polymerase activity could be detected *in vitro* using subcellular fractions of *P. aeruginosa*. Evidence was provided that an envelope preparation containing cytoplasmic membrane, outer membrane, and associated proteins constitute the alginate polymerase complex, which was in addition stabilized by the nonionic detergent Triton-X100 (Table 2). This was further confirmed by separation of cytoplasmic membrane and outer membrane, which led to inactivation of alginate polymerase. These findings also support previous suggestions (16, 23, 49) of a protein scaffold in the periplasm related to alginate polymerisation and/or modification. Additionally, these data suggest that the outer membrane export protein AlgE is attached to the protein scaffold composed of cytoplasmic membrane proteins (Alg8 and Alg44) and periplasmic proteins (AlgK, AlgL, AlgG, and AlgX). Thus, we propose that alginate polymerisation and

export through the outer membrane are coordinated via the formation of a protein complex involving cytoplasmic and outer membrane proteins, as well as periplasmic proteins.

In previous studies, only transposon mutagenesis has been used to characterize the putative *alg8* gene within the alginate biosynthesis operon and DNA fragments comprising more than one open reading frame (ORF) were applied for complementation studies (31, 63). The transposon insertions showed polar effects on other biosynthesis genes (6, 63). Thus, to evaluate the requirement of the designated ORF of *alg8* (31), we generated a marker-free, nonpolar *alg8* deletion mutant by using homologous recombination (Fig. 1). The mutant *P. aeruginosa* PDO300Δalg8 showed a nonmucoid phenotype but, in contrast to the also-reported nonmucoid phenotypes of *algK*, *algG* and *algX* deletion mutants, suggested to be caused by degradation of the nascent alginate chain by alginate lyase AlgL (23, 49), we were unable to detect uronic acid oligomers (< 5,000 Da) or monomers in the respective culture supernatants. Jain et al. (23) suggested that the proteins AlgK and AlgG were part of a scaffold surrounding and therefore protecting the nascent alginate chain, and the findings of Robles-Price et al. (49) suggested that AlgX is also involved in the scaffold formation. The culture supernatants of the respective mutants contained unsaturated uronic acid oligomers, which indicated alginate lyase degradation. Since no uronic acid mono- or oligomers have been found in the supernatant of the *alg8* deletion mutant, our findings suggest that deletion of *alg8* abolishes alginate polymerisation. Further studies will reveal whether alginate production deficiency is caused by a lack of polymerisation or membrane translocation of the precursor GDP-mannuronic acid. Many glycosyltransferases of the class II are involved not only in membrane translocation but also in polymerisation itself (52, 53). Thus, Alg8 might be the catalytic subunit of the alginate polymerase as previously suggested (45). This hypothesis was supported by the 20-fold overproduction of alginate by *P. aeruginosa* PDO300Δalg8 (pBBR1MCS-5:alg8) compared to *P. aeruginosa* PDO300, indicating that Alg8 is the bottleneck in alginate production (Table 3). Unlike previous studies (31, 63), we used a defined PCR fragment comprising only the designated *alg8* ORF of nonmucoid *P. aeruginosa* PAO1 and not DNA fragments that originated from subcloning of the alginate biosynthesis operon of *P. aeruginosa* FRD1. We were therefore able to limit the complementing DNA sequence to the defined ORF of Alg8.

Additional copy numbers of *alg8* enhanced the alginate production of strain *P. aeruginosa* PDO300 by a factor of 15. *P. aeruginosa* PDO300 was used for further complementation studies because of plasmid stability problems associated with clinical alginate-overproducing isolate *P. aeruginosa* FRD1. Recent publications demonstrate that strain *P. aeruginosa* FRD1:pJLS3, in which the alginate biosynthesis operon is under control of the strong IPTG-inducible P*tac* promoter, produced 0.55 g of alginate per g of cellular dry weight (1). Comparison of these

alginate production data with the alginate production of strain *P. aeruginosa* PDO300Δalg8 pBBR1MCS-5:alg8, which produced ca. 2.5 g of alginate per g of cellular dry weight, suggests that Alg8 is the bottleneck in alginate production. This enabled us to functionally assign the ORF of *alg8* as a complementary unit to restore alginate production in the *alg8* deletion mutant.

Interestingly, not only was the alginate production of the complemented mutant *P. aeruginosa* PDO300Δalg8 (pBBR1MCS-5:alg8) strongly enhanced but also the polymer composition was altered as indicated by ^{1}H-NMR analysis. Overproduction of Alg8 seems to influence polymer composition and properties. The ^{1}H-NMR data indicated a slightly increased degree of acetylation, whereas the amount of guluronic acid residues was found to be significantly reduced. Further experiments are required to explain how Alg8 impacts on polymer acetylation and composition.

Fusion protein analysis and the predicted topology model suggest that Alg8 is a transmembrane protein, with a N-terminal signal sequence and four transmembrane helices. The C terminus is presumably located in the periplasm, as indicated by alkaline phosphatase activity of the respective fusion protein. Since the C terminus appears to be very hydrophobic, we suggest that the untagged C-terminal end just crosses the cytoplasmic membrane and/or stays embedded in the cytoplasmic membrane. Our Alg8 protein model prediction suggests a large cytosolic loop at the N terminus (Fig. 2). This N-terminal domain shares homology with class II β-glycosyltransferases enabling development of a structural model of Alg8 based on the known structure of glycosyltransferase SpsA. Moreover, the cytosolic localization of the putative active site is consistent with the availability of GDP-mannuronic acid in the cytosol. Cellular fractionation experiments with the alkaline phosphatase fusion protein of Alg8 and analysis of specific alkaline phosphatase activity suggested localization in the cytoplasmic membrane. Although PhoA assays of the different cellular fractions showed PhoA activity, the highest specific activity was found to be associated with the cytoplasmic membrane fraction. The structural model of Alg8 indicated that the proposed residues Asp 161, Asp 250 and Cys 215 are located in or adjacent to the core structure (Fig. 2). These residues might be involved in substrate binding, because homologous amino acids are responsible for the nucleotide-sugar binding in SpsA (4). These and other amino acids that are proposed to be involved in catalytic function are currently investigated by site-specific mutagenesis. The identification of catalytic residues might shed light into the alginate polymerisation reaction and might enable the design of inhibitors that are able to block polymerisation and therefore impair biofilm formation in cystic fibrosis patients. Furthermore, inhibitors of alginate polymerisation could be identified using the *in vitro* alginate synthesis assay as screening tool.

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References

1. **Albrecht, M. T., and N. L. Schiller.** 2005. Alginate lyase (AlgL) activity is required for alginate biosynthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **187:**3869-3872.
2. **Bernsel, A., and G. Von Heijne.** 2005. Improved membrane protein topology prediction by domain assignments. Protein Sci. **14:**1723-1728.
3. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484-489.
4. **Charnock, S. J., and G. J. Davies.** 1999. Structure of the nucleotide-diphospho-sugar transferase, SpsA from *Bacillus subtilis*, in native and nucleotide-complexed forms. Biochemistry **38:**6380-6385.
5. **Chitnis, C. E., and D. E. Ohman.** 1993. Genetic analysis of the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* shows evidence of an operonic structure. Mol. Microbiol. **8:**583-593.
6. **Darzins, A., and A. M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. **159:**9-18.
7. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15:**4567-4581.
8. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. J. Bacteriol. **176:**2773-2780.
9. **Ethier, J., and J. M. Boyd.** 2000. Topological analysis and role of the transmembrane domain in polar targeting of PilS, a *Pseudomonas aeruginosa* sensor kinase. Mol. Microbiol. **38:**891-903.
10. **Evans, L. R., and A. Linker.** 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. **116:**915-924.

11. **Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman.** 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. J. Bacteriol. **176:**1821-1830.
12. **Franklin, M. J., and D. E. Ohman.** 1993. Identification of *algF* in the alginate biosynthetic gene cluster of *Pseudomonas aeruginosa* which is required for alginate acetylation. J. Bacteriol. **175:**5057-5065.
13. **Franklin, M. J., and D. E. Ohman.** 1996. Identification of *algI* and *algJ* in the *Pseudomonas aeruginosa* alginate biosynthetic gene cluster which are required for alginate O acetylation. J. Bacteriol. **178:**2186-2195.
14. **Franklin, M. J., and D. E. Ohman.** 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. J. Bacteriol. **184:**3000-3007.
15. **Gacesa, P., and N. J. Russell.** 1990. The structure and property of alginate, p. 29-49, *Pseudomonas* Infection and Alginates. Chapman & Hall, London.
16. **Gimmestad, M., H. Sletta, H. Ertesvåg, K. Bakkevig, S. Jain, S.-J. Suh, G. Skjåk-Bræk, T. E. Ellingsen, D. E. Ohman, and S. Valla.** 2003. The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J. Bacteriol. **185:**3515-3523.
17. **Goldberg, J. B., K. Hatano, and G. B. Pier.** 1993. Synthesis of lipopolysaccharide O side chains by *Pseudomonas aeruginosa* PAO1 requires the enzyme phosphomannomutase. J. Bacteriol. **175:**1605-1611.
18. **Grasdalen, H., B. Larsen, and O. Smidsrod.** 1979. A P.M.R. study of the composition and sequence of uronate residues in alginates. Carbohydr. Res. **68:**23-31.
19. **Gutierrez, C., and J. C. Devedjian.** 1989. A plasmid facilitating *in vitro* construction of *phoA* gene fusions in *Escherichia coli*. Nucleic Acids Res. **17:**3999.
20. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.
21. **Hoffmann, N., A. A. Amara, B. B. Beermann, Q. Qi, H. J. Hinz, and B. H. Rehm.** 2002. Biochemical characterization of the *Pseudomonas putida* 3-hydroxyacyl ACP:CoA transacylase, which diverts intermediates of fatty acid de novo biosynthesis. J. Biol. Chem. **277:**42926-42936.
22. **Holloway, B. W., H. Matsumoto, and P. V. Phibbs, Jr.** 1986. The chromosome map of *Pseudomonas aeruginosa* PAO. Acta Microbiol. Pol. **35:**161-164.

23. **Jain, S., M. J. Franklin, H. Ertesvåg, S. Valla, and D. E. Ohman.** 2003. The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol. Microbiol. **47:**1123-1133.
24. **Jain, S., and D. E. Ohman.** 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J. Bacteriol. **180:**634-41.
25. **Kall, L., A. Krogh, and E. L. Sonnhammer.** 2004. A combined transmembrane topology and signal peptide prediction method. J. Mol. Biol. **338:**1027-1036.
26. **Karchin, R., M. Cline, Y. Mandel-Gutfreund, and K. Karplus.** 2003. Hidden Markov models that use predicted local structure for fold recognition: alphabets of backbone geometry. Proteins: Structure, Function, and Genetics **51:**504-514.
27. **Kelley, L. A., R. M. MacCallum, and M. J. Sternberg.** 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J. Mol. Biol. **299:**499-520.
28. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, 2nd, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-176.
29. **Linker, A., and R. S. Jones.** 1966. A new polysaccharide resembling alginic acid isolated from pseudomonads. J. Biol. Chem. **241:**3845-3851.
30. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194-222.
31. **Maharaj, R., T. B. May, S. K. Wang, and A. M. Chakrabarty.** 1993. Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene **136:**267-269.
32. **Manoil, C.** 1991. Analysis of membrane protein topology using alkaline phosphatase and beta-galactosidase gene fusions. Methods Cell. Biol. **34:**61-75.
33. **Martin, D. W., M. J. Schurr, M. H. Mudd, J. R. Govan, B. W. Holloway, and V. Deretic.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proc. Natl. Acad. Sci. USA **90:**8377-8381.
34. **Mathee, K., C. Sternberg, O. Ciofu, P. Jensen, J. Campbell, M. Givskov, D. E. Ohman, N. Hoiby, S. Molin, and A. Kharazmi.** 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiol. **145:**1349-1357.
35. **Mejia-Ruiz, H., J. Guzman, S. Moreno, G. Soberon-Chavez, and G. Espin.** 1997. The *Azotobacter vinelandii alg8* and *alg44* genes are essential for alginate synthesis and can be transcribed from an *algD*-independent promoter. Gene **199:**271-277.

36. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
37. **Moller, S., M. D. Croning, and R. Apweiler.** 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics **17:**646-653.
38. **Monday, S. R., and N. L. Schiller.** 1996. Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX. J. Bacteriol. **178:**625-632.
39. **Nivens, D. E., D. E. Ohman, J. Williams, and M. J. Franklin.** 2001. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J. Bacteriol. **183:**1047-1057.
40. **Ohman, D. E., and A. M. Chakrabarty.** 1981. Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. Infect. Immun. **33:**142-148.
41. **Pedersen, S. S., N. Hoiby, F. Espersen, and C. Koch.** 1992. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax **47:**6-13.
42. **Pier, G. B., F. Coleman, M. Grout, M. Franklin, and D. E. Ohman.** 2001. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. Infect. Immun. **69:**1895-1901.
43. **Pindar, D. F., and C. Bucke.** 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. Biochem. J. **152:**617-622.
44. **Preiss, J.** 1966. GDP-Mannose-dehydrogenase from Arthrobacter. Methods Enzymol. **8:**285-287.
45. **Rehm, B. H.** A. 2002. Alginates from Bacteria, p. 179-211. *In* E. J. Vandamme, S. DeBaets, and A. Steinbüchel (ed.), Biopolymers, vol. Polysaccharides. Wiley VCH.
46. **Rehm, B. H. A.** 1998. Alginate lyase from *Pseudomonas aeruginosa* CF1/M1 prefers the hexameric oligomannuronate as substrate. FEMS Microbiol. Lett. **165:**175-180.
47. **Rehm, B. H. A.** 1996. The *Azotobacter vinelandii* gene *algJ* encodes an outer membrane protein presumably involved in export of alginate. Microbiology **142:**873-880.
48. **Rehm, B. H. A., G. Boheim, J. Tommassen, and U. K. Winkler.** 1994. Overexpression of *algE* in Escherichia coli: subcellular localization, purification, and ion channel properties. J. Bacteriol. **176:**5639-5647.
49. **Robles-Price, A., T. Y. Wong, H. Sletta, S. Valla, and N. L. Schiller.** 2004. AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **186:**7369-7377.

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

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

52. **Saxena, I. M., and R. M. Brown, Jr.** 2000. Cellulose synthases and related enzymes. Curr. Opin. Plant. Biol. **3:**523-531.

53. **Saxena, I. M., R. M. Brown, Jr., and T. Dandekar.** 2001. Structure-function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57:**1135-1148.

54. **Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia, and B. Henrissat.** 1995. Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. J. Bacteriol. **177:**1419-1424.

55. **Schiller, N. L., S. R. Monday, C. M. Boyd, N. T. Keen, and D. E. Ohman.** 1993. Characterization of the *Pseudomonas aeruginosa* alginate lyase gene (algL): cloning, sequencing, and expression in *Escherichia coli*. J. Bacteriol. **175:**4780-4789.

56. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. Arch. Mikrobiol. **38:**209-222.

57. **Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting.** 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA **95:**5857-5864.

58. **Schurks, N., J. Wingender, H. C. Flemming, and C. Mayer.** 2002. Monomer composition and sequence of alginates from *Pseudomonas aeruginosa*. Int. J. Biol. Macromol. **30:**105-111.

59. **Schurr, M. J., D. W. Martin, M. H. Mudd, N. S. Hibler, J. C. Boucher, and V. Deretic.** 1993. The *algD* promoter: regulation of alginate production by *Pseudomonas aeruginosa* in cystic fibrosis. Cell. Mol. Biol. Res. **39:**371-376.

60. **Shankar, S., R. W. Ye, D. Schlictman, and A. M. Chakrabarty.** 1995. Exopolysaccharide alginate synthesis in *Pseudomonas aeruginosa*: enzymology and regulation of gene expression. Adv. Enzymol. Relat. Areas Mol. Biol. **70:**221-255.

61. **Simon, R., U. Priefer, and A. Pühler.** 1983. Bio-Technology **1:**784-791.

62. **Skjåk-Bræk, G., H. Grasdalen, and B. Larsen.** 1986. Monomer sequence and acteylation pattern in some bacterial alginates. Carbohydr. Res. **154:**239-250.

63. **Wang, S. K., I. Sa'-Correia, A. Darzins, and A. M. Chakrabarty.** 1987. Characterization of the *Pseudomonas aeruginosa* alginate (alg) gene region II. J Gen Microbiol **133:**2303-2314.

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

Chapter III

Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*

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Abstract

Here the putative alginate biosynthesis gene *alg44* of *Pseudomonas aeruginosa* was functionally assigned. Nonpolar isogenic *alg44* deletion mutants of *P. aeruginosa* were generated and did neither produce alginate nor released free uronic acids. No evidence for alginate enrichment in the periplasm was obtained. Alginate production was restored by introducing only the gene *alg44*. PhoA fusion protein analyses suggested that Alg44 is a soluble protein localized in the periplasm. Hexahistidine-tagged Alg44 was detected by immunoblotting. The corresponding 42.6 kDa protein was purified and identified by MALDI-TOF/MS analysis. Alg44 might be directly involved in alginate polymerisation presumably by exerting a regulatory function.

Introduction

Bacterial alginates consist of β-1,4-linked monomers of β-D-mannuronic acid and its C5 epimer α-L-guluronic acid. Alginate is an important virulence factor produced by the opportunistic human pathogen *Pseudomonas aeruginosa*. Primarily synthesized as polymannuronate from GDP-mannuronic acid, the nascent polymer chain undergoes modification in the periplasm by acetylation and epimerization [1]. A protein scaffold might guide the alginate chain through the periplasm towards the outer membrane export channel [2-5]. Although initial alginate polymerisation and cytoplasmic membrane transfer are still not understood, it has been recently shown that Alg8 seems to be a bottleneck in alginate production. Increased copy number of *alg8* resulted in an increased alginate production, suggesting that the putative glycosyltransferase Alg8 is a catalytic subunit of the alginate polymerase [6]. So far only the isogenic *alg8* deletion mutant did neither produce alginate nor released uronic acids.

In this study, the *alg44* gene localized in the alginate biosynthesis gene cluster of *P. aeruginosa* was functionally assigned by generation of a nonpolar *alg44* deletion mutant, functional expression as well as by subcellular localization, purification and identification of the Alg44 protein.

Materials and Methods

Bacterial strains and growth conditions.

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* strains were cultivated as previously described [6]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of isogenic alg44 deletion mutants.

Two regions of the *alg44* gene were amplified using *Taq* polymerase and primers alg441N-Ec5, alg441N-Ba, alg442C-Ba and alg442C-Ec5 (Table 1). Region alg44N (362 bp) comprises the first 358 nucleotides of the designated *alg44* open reading frame [7] including 4 nucleotides upstream of the start codon. Region alg44C (332 bp) comprised bases 820-1151 relative to the *alg44* coding region [7], respectively. Both PCR products represented the flanking region for homologous recombination and isogenic mutants were generated and confirmed as previously described [6].

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

Strains, plasmids or oligonucleotides	Description	Source or reference
Strains		
P. aeruginosa		
FRD1	Cystic fibrosis isolate; Alg^+	[31]
FRDΔalg44Gm	Δ*alg44*::*aacC1*; Alg^-	This study
FRDΔalg44	Δ*alg44*; Alg^-	This study
PAO1	Prototrophic wild-type strain; Alg^-	[32]
PDO300	Δ*mucA22* variant of PAO1; Alg^+	[33]
PDO300Δalg44Gm	Δ*alg44*::*aacC1*; Alg^-	This study
PDO300Δalg44	Δ*alg44*; Alg^-	This study
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi*-1; *proA*, *hsd*R*17* (r_k^-, m_k^+), *recA1*, *tra*-gene of plasmid RP4 integrated in chromosome	[34]
MC4100	F– *araD*139 Δ(*argF-lac*)U169 *rpsL*150 (Str^r) *relA1 flb*B5301 *deoC*1 *ptsF*25 *rbs*R	[35]
DH5α	F´/*endA1 hsd*R*17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA argF*) U169 *deo*R (Φ80*dlac*Δ(*lacZ*)M15)	Life Technologies
BL21(DE3) pLysS	F– *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cm^r)	Novagen
Plasmids		
pBBR1MCS-5	Gm^r; Broad-host-range vector; $P_{(lac)}$	[8]
pBBR1MCS-5:alg44	*Hin*dIII-*Bam*HI fragment comprising gene *alg44* inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44His	*Hin*dIII-*Bam*HI fragment encoding C-terminally hexahistidine-tagged Alg44 inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44(Δstop)	*Hin*dIII-*Bam*HI fragment encoding Alg44 without stop codon inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44lacZ	Translational Alg44-LacZ fusion, inserted into vector pBBR1MCS-5	This study
pBBR1MCS-5:alg44phoA	Translational Alg44-PhoA fusion, inserted into vector pBBR1MCS-5	This study
pEX100T	Ap^r; Cb^r, gene replacement vector containing *sacB* gene for counterselection	[36]
pEX100TΔalg44Gm	Ap^r, Cb^r, Gm^r; vector pEX100T with *Sma*I inserted *alg44* deletion construct	This study
pPS856	Ap^r; Gm^r; source of 1100 bp *Bam*HI-fragment comprising *aacC1* gene flanked by FRT signal sequences	[36]
pPFLP2	Ap^r; Cb^r; broad-host-range vector encoding Flp recombinase	[36]
pPHO7	Ap^r; *phoA* without signal sequence	[9]
pJE608	LacZ lacking the first 8 amino acids with promoter $P_{(tac)}$ in pMMB67EH	[10]
pT7-7	Ap^r, T7Φ10 expression vector	[14]
pT7-7:alg44His	*Nde*I-*Hin*dIII fragment of pBBR1MCS-5:alg44His inserted into vector pT7-7	This study
Oligonucleotides		
alg441N-Ec5	GCGTCGATATCCACCATGAATACAGCCGTCAACG	
alg441N-Ba	TCACGGATCCCCAGGTAGGAGGTGATCAGGTAG	
alg442C-Ba	CTACGGATCCCAACTGGTAGCCGACGGGCAATAC	
alg442C-Ec5	GTGTCGATATCGTCACGGCCTTGTTCAGCAG	
alg44up	TGATGGATCCGTTCACCATGCTGGTGCTGTTC	
alg44down	AACTCTGCAGCAGGCTGACGGTG	

Table 1: (Cont) Bacterial strains, plasmids and oligonucleotides used in this study

Strains, plasmids or oligonucleotides	Description	Source or reference
Oligonucleotides		
alg44N(HiSDNd)	CCGCCAAGCTTAGGAGCCCGACCATATGAATACAGCCGTCAACGTCAACG	
alg44C(Ba)	AACGGATCCTCAGCGAGCGGTGGCCAGGGTCAC	
alg44C(HisBa)	AACGGATCCTCAATGGTGATGGTGATGGTGACGAGCGGTGGCCAGGGTCAC	
alg44C(Δstop)	AAAAAGGATCCCGAGCGGTGGCCAGGGTCAC	

Complementation of isogenic alg44 deletion mutants.

For complementation of *alg44* deletion mutants, the coding region of the *alg44* gene of *P. aeruginosa* PAO1 was amplified by PCR using primers alg44N(HiSDNd) and alg44C(Ba) and subcloned into broad-host-range vector pBBR1MCS-5 [8], resulting in plasmid pBBR1MCS-5:alg44. Additionally, the 3'-end primer alg44C(HisBa) was used to generate an *alg44* gene encoding a C-terminally hexahistidine-tagged Alg44, which was inserted into vector pBBR1MCS-5 as described above (Table 1). All inserts cloned into the multiple cloning site of vector pBBR1MCS-5 are under control of the *lac* promoter.

Subcellular localization of Alg44 using either PhoA or LacZ fusions.

The gene *alg44* was amplified by PCR using *Pfx* polymerase and primers alg44N(HiSDNd) and alg44C(Δstop). The corresponding 1202 bp PCR product was used to construct plasmid pBBR1MCS-5:alg44(Δstop). *Xba*I-*Bam*HI fragments of vectors pPHO7 [9] and pJE608 [10] were inserted into *Xba*I-*Bam*HI restricted pBBR1MCS-5:alg44(Δstop) to generate translational fusions with either PhoA or LacZ. Subcellular fractionation was performed as described previously [6]. Osmotic shock was used to isolate periplasmic extracts [11].

Alkaline phosphatase / β-galactosidase activity assays.

Alkaline phosphatase and β-galactosidase enzymatic assays were performed according to the methods of Miller [12] and Manoil [13], respectively.

Heterologous production and purification of Alg44 in *E. coli*.

Plasmid pBBR1MCS-5:alg44His was hydrolyzed with *Nde*I and *Bam*HI and the resulting 1.2 kbp fragment was cloned into *Nde*I and *Bam*HI hydrolyzed vector pT7-7 [14], creating plasmid pT7-7:alg44His. *E. coli* BL21 (DE3) pLysS was used for heterologous expression. Crude extracts were subjected to immobilized metal ion chromatography (Ni-NTA agarose, Qiagen) using denaturing conditions (6 M GuHCl). The hexahistidine-tagged protein was purified using buffers as described in the manufacturers manual (Qiagen).

SDS-PAGE and immunoblot analyses.

Proteins from *P. aeruginosa* or *E. coli* containing plasmids encoding Alg44His$_6$ were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) [15]. Proteins were electroblotted onto nitrocellulose membrane (Protran BA 83, Schleicher & Schuell) and then incubated with HisProbe™-horseradish peroxidase conjugate (HisProbe™-HRP, Pierce). Immunoblots were developed using a chemiluminescence protocol according to the manufacturers manual (SuperSignal® West HisProbe™, Pierce).

Alginate production and uronic acid assays.

Alginate production assays and uronic acid assays were performed as described previously [6].

Electron microscopy.

Cells were fixed as described elsewhere [16], and electron microscopy was performed on a Philips CM201c Transmission Electron Microscope [17].

Results

Construction of an isogenic alg44 knock out mutant.

To investigate the function of the *alg44* gene in alginate biosynthesis by *P. aeruginosa*, marker-free and nonpolar *alg44* deletion mutants of alginate-overproducing strains *P. aeruginosa* PDO300 and *P. aeruginosa* FRD1 were generated, respectively (Fig. 1).

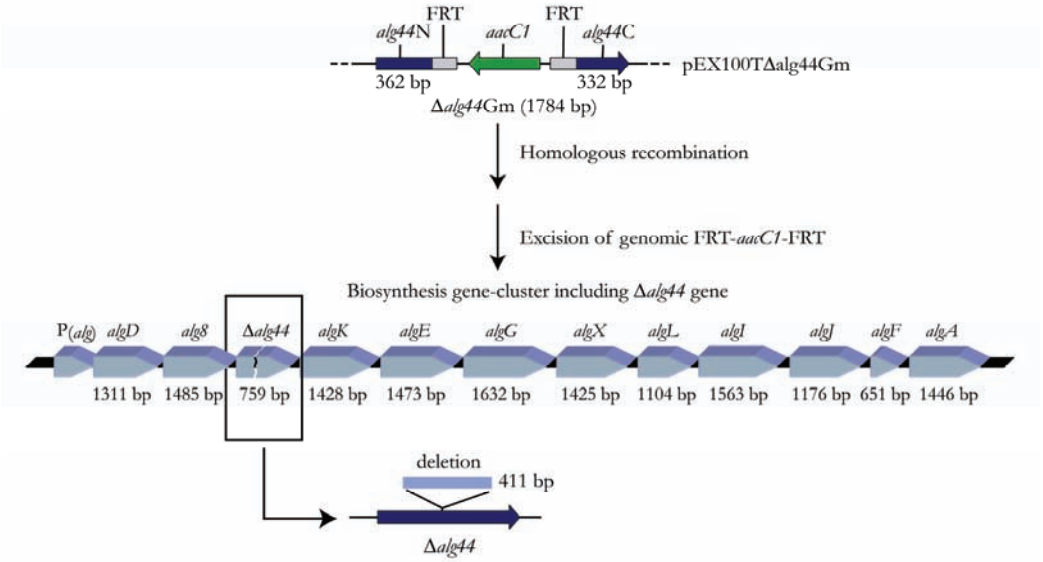

Figure 1: **Schematic view of *alg44* knock out construct of plasmid pEX100TΔalg44Gm used for homologous recombination and the alginate biosynthesis operon after replacement of native *alg44* gene with *Δalg44*.**

Both deletion mutants showed a non-mucoid and alginate-negative phenotype when cultivated on agar plates. To investigate whether the non-mucoid phenotype is caused by alginate lyase (AlgL) mediated degradation of an exposed unprotected alginate chain, culture supernatants of *P. aeruginosa* PDO300Δalg44 were analysed with respect to alginate and free uronic acids. Neither alginate nor free uronic acids were detected (Table 2). Recently, Jain and Ohman showed that alginate lyase is required for alginate production and that AlgL deficient mutants accumulated alginate in the periplasm [4]. No uronic acids were detected when analyzing periplasmic extract of the mutant, and electron microscopy analysis of cells of *P. aeruginosa* PDO300 wild type and *alg44* deletion mutant showed neither swelling of the periplasm nor differences in cell shape and structure between wild type and mutant (data not shown).

Table 2: Alginate and cellular dry mass production by different *P. aeruginosa* strains harbouring various plasmids

Strain	Mean ± S.D.	
	Alginate production (g/g CDM)	Cellular dry mass (CDM [g])
PDO300	0.131 ± 0.037	0.171 ± 0.027
PDO300 (pBBR1MCS-5)	0.629 ± 0.026	0.196 ± 0.006
PDO300 (pBBR1MCS-5:alg44)	0.731 ± 0.122	0.171 ± 0.015
PDO300Δalg44	ND[α]	0.127 ± 0.006
PDO300Δalg44 (pBBR1MCS-5)	ND[α]	0.173 ± 0.003
PDO300Δalg44(pBBR1MCS-5:alg44)	1.187 ± 0.721	0.158 ± 0.034
PDO300Δalg44(pBBR1MCS-5:alg44His)	0.786 ± 0.257	0.159 ± 0.124
PDO300Δalg44(pBBR1MCS-5:alg44lacZ)	0.392 ± 0.031	0.157 ± 0.003
PDO300Δalg44(pBBR1MCS-5:alg44phoA)	1.343 ± 0.914	0.145 ± 0.063

[α] ND, not detectable. *P* values were determined using Kruskal-Wallis one way analysis of variance on ranks. All mean values and S.D.s were based on 4 independent experiments.

Restoration of alginate biosynthesis using the *alg44* open reading frame.

To verify that disruption of *alg44* had no downstream effects on the alginate biosynthesis operon and to functionally assign the putative *alg44* gene, a plasmid containing only the designated *alg44* ORF (pBBR1MCS-5:alg44) was used to complement the *P. aeruginosa* PDO300Δalg44 mutant. This plasmid mediated restoration of alginate production in the *alg44* mutant. Alginate production of complemented *alg44* mutants carrying either the native *alg44* gene or a gene encoding C-terminally tagged Alg44 in vector pBBR1MCS-5, had no statistically significant influence ($P>0.05$) on alginate yield (Table 2). Since *P. aeruginosa* PDO300 harbouring gentamycin-resistance mediating vector pBBR1MCS-5 showed already a presumably antibiotic stress mediated increased alginate production when compared with the wild type strain; this recombinant strain was used as control (Table 2). The cellular dry mass (CDM) produced by the various strains showed no significant difference ($P>0.05$) (Table 2).

Analysis of translational fusion proteins of Alg44.

Topology prediction analysis (SMART [18], TMHMM [19]) of Alg44 suggest one transmembrane domain (amino acids 159-178) with the N terminus exposed to the cytosol. No signal sequence could be predicted [18,19]. To investigate the localization and topology of Alg44, C-terminal translational fusions to reporter enzymes LacZ and PhoA were generated, respectively. Both reporter enzyme fusions had no impact on Alg44 function, as indicated by complementation studies (Table 2). Reporter enzyme assays revealed a specific alkaline phosphatase activity of 5.036 ± 0.648 U/mg of CDM and a β-galactosidase activity of 0.834 ± 0.215 U/mg, respectively. The reporter enzyme assays were also performed in strains of *E. coli* DH5α and MC4100, revealing an alkaline phosphatase activity of 2.973 ± 0.282 U/mg CDM and a β-galactosidase activity of 0.906 ± 0.112 U/mg CDM. Cellular fractionation experiments were performed with strain *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5:alg44phoA) in order to investigate the subcellular localization of Alg44. Interestingly, higher alkaline phosphatase activity was detected in the soluble supernatant, but not in the insoluble membrane fraction. The membrane free supernatant revealed an alkaline phosphatase activity of 17.577 ± 1.843 U/mg protein, whereas the membrane fraction showed only an activity of 4.086 ± 0.510 U/mg protein. Isolated periplasmic extract showed an alkaline phosphatase activity of 8.302 ± 0.720 U/mg protein.

Immunological detection and purification of Alg44His$_6$.

To detect and verify the expression of *alg44* and to demonstrate the presence of Alg44, the production of hexahistidine-tagged Alg44 protein was investigated in the native host *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5:alg44His) as well as in *E. coli* BL21 (pLysS, pT7-7:alg44His). Alg44His$_6$ was detected by immunoblotting using anti-His$_6$-antibodies. The immunoblot showed specific antibody binding to a protein with the apparent molecular weight of 42.4 ± 0.4 kDa, which was consistent with the predicted molecular weight of Alg44His$_6$ of 42.6 kDa (Fig. 2A and B). To verify that the detected protein is encoded by the designated *alg44* ORF, crude extracts of *E. coli* BL21 (pLysS, pT7-7:alg44His) were subjected to affinity chromatography. SDS-PAGE and MALDI-TOF/MS analysis showed that Alg44His$_6$ could be purified from crude extracts (Fig. 2C).

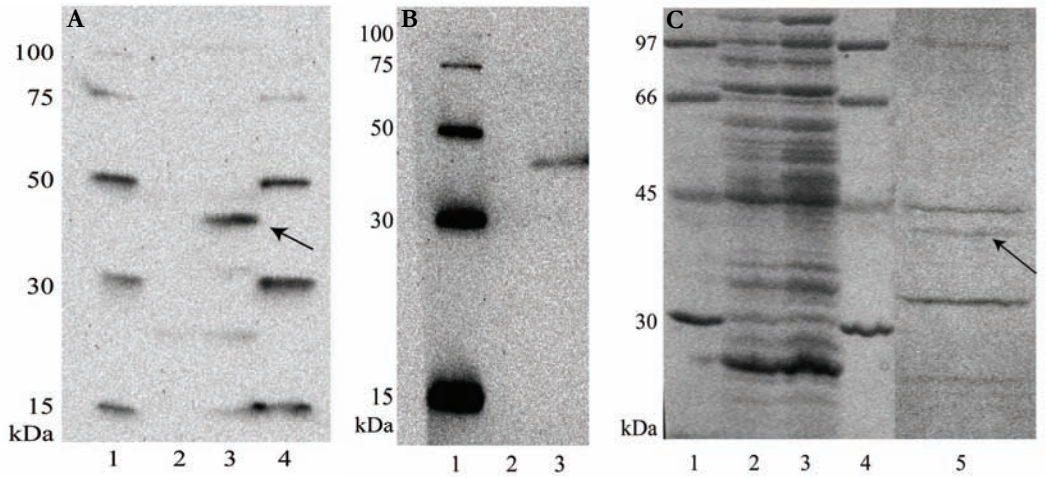

Figure 2: **(A) Immunoblot analysis of cell extracts of *E. coli* BL21 (pLysS) harbouring overexpression plasmids. Lane 1 and 4, hexahistidine tagged molecular weight standard; lane 2, *E. coli* BL21 (pLysS) (pT7-7); lane 3, *E. coli* BL21 (pLysS) (pT7-7:alg44His); Arrow indicates Alg44His$_6$. (B) Immunoblot analysis of cell extracts of *P. aeruginosa* PDO300Δalg44 harbouring various plasmids. Lane 1, hexahistidine tagged molecular weight standard; lane 2, *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5); lane 3, *P. aeruginosa* PDO300Δalg44 (pBBR1MCS-5:alg44His). (C) SDS-PAGE analysis of Alg44His$_6$ purified by affinity chromatography. Lane 1 and 4, molecular weight standard; lane 2, crude extract of *E. coli* BL21 (pLysS) (pT7-7); lane 3, crude extract of *E. coli* BL21 (pLysS) (pT7-7:alg44His); lane 5, partially purified Alg44His$_6$; Arrow indicates protein subjected to MALDI-TOF/MS analysis.**

Discussion

Here the first marker-free nonpolar *alg44* deletion mutant of *P. aeruginosa* was generated by using homologous recombination and the loss of alginate production suggested that Alg44 is required for alginate production (Fig. 1, Table 2). Uronic acid monomer and oligomer analysis suggested that the alginate-negative phenotype was not due to extensive degradation of alginate by the alginate lyase as reported for *algG*, *algK* and *algX* mutants [20-22] and that Alg44 is involved in alginate polymerisation. AlgG, AlgK and AlgX are proposed periplasmic scaffold proteins. AlgX might also interact with a serine-protease homologue MucD, which is involved in alginate biosynthesis gene regulation [23]. *P. aeruginosa* PDO300Δalg44 did not accumulate alginate in the periplasm, as was found in *algL* mutants [4].

Only the 1170 bp ORF of *alg44* [24] encoding a putative 41.78 kDa protein mediated restoration of wildtype level alginate production suggesting that *alg44* encodes a protein required for alginate production. Production of Alg44 with the corresponding molecular weight was confirmed by immunoblot analysis of the hexahistidine-tagged Alg44, which was also purified and identified by MALDI-TOF/MS analysis.

C-terminal fusions to Alg44 did not interfere with protein functionality, suggesting that the C terminus is not directly involved in Alg44 function (Table 2). The activity of the alkaline phosphatase and the lack of β-galactosidase activity supported the constrained topology

prediction using HMM-based topology tool Phobius [25], which suggested a cytosolic N terminus, a transmembrane domain (amino acid 159 – 178) and a periplasmic localization of the C terminus. However, the highest specific alkaline phosphatase activity was not associated with the insoluble membrane, but with the soluble membrane-free fraction as well as the periplasmic extract, which suggested a periplasmic localization of Alg44. This finding did not support the presence of the predicted transmembrane domain. Protein export into the periplasm is usually depended on a N terminal signal sequence. The detection of 42.6 kDa hexahistidine-tagged protein Alg44 in *P. aeruginosa* indicated the presence of an unmodified mature protein (Fig. 2), which is consistent with the lack of a signal sequence. Other proteins such as, e.g., the dehalogenases LinA and LinB from pseudomonads/sphingomonads, have been localized to the periplasm without N-terminal processing [26].

Homology searches [27] revealed a 16.8 % similarity (Jscore: 106.71) of the C-terminal half of Alg44 (amino acids 202-389) to MexA (PDB structure 1t5eA/1vf7a). MexA is a periplasmic membrane fusion protein (MFP) component of the multidrug transporter complex MexAB-OprM in *P. aeruginosa*, linking the cytoplasmic membrane component MexB with the outer membrane export protein OprM [28]. These similarities together with the periplasmic localization of protein Alg44 might indicate a role of Alg44 bridging the cytoplasmic-membrane associated polymerase (only protein shown to be in the cytoplasmic membrane is Alg8) with the outer membrane export channel AlgE (Fig. 3). The C-terminal part of Alg44 might be involved in connecting or colocalizing Alg8 and AlgE, but scaffold proteins AlgKGXL might be required to protect the nascent alginate chain against degradation. Recently, a PilZ domain (PDB structure 1yln / 1ywu) in the N-terminal region of Alg44 was identified [29], which might be involved in binding the novel regulatory molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) [30]. Thus Alg44 might function as a regulatory membrane fusion protein (Fig. 3).

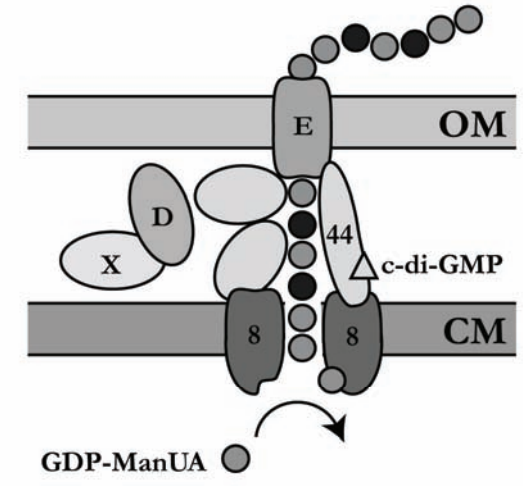

Figure 3: **Model of the alginate polymerisation, modification and export. CM, cytoplasmic membrane; OM, outer membrane, 8, Alg8; 44, Alg44, E, AlgE; X, AlgX; D, MucD; c-di-GMP, bis-(3'-5')-cyclic dimeric guanosine monophosphate; GDP-ManUA, GDP-mannuronic acid.**

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References

[1] **Rehm, B.H.A. and Valla, S. (1997).** Bacterial alginates: biosynthesis and applications. Appl. Microbiol. Biotechnol. **48**: 281-288.

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

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

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

[5] **Gimmestad, M., Sletta, H., Ertesvåg, H., Bakkevig, K., Jain, S., Suh, S.-J., Skjåk-Bræk, G., Ellingsen, T.E., Ohman, D.E. and Valla, S. (2003).** The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J. Bacteriol. **185**: 3515-3523.

[6] **Remminghorst, U. and Rehm, B.H.A. (2006).** *In vitro* Alginate Polymerisation and the Functional Role of Alg8 in Alginate Production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **72**: 298-305.

[7] **Maharaj, R., May, T.B., Wang, S.K. and Chakrabarty, A.M. (1993).** Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene **136**: 267-269.

[8] **Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd and Peterson, K.M. (1995).** Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166**: 175-176.

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

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

[11] **Dvorak, H.F., Brockman, R.W. and Heppel, L.A. (1967).** Purification and properties of two acid phosphatase fractions isolated from osmotic shock fluid of *Escherichia coli*. Biochemistry **6**: 1743-1751.

[12] **Miller, J.H. (1972).** Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

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

[14] **Tabor, S. and Richardson, C.C. (1985).** A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**: 1074-1078.

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

[16] **Karnovski (1965).** A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell. Biol. **27**: 137.

[17] **Venable, J.H. and Coggeshall, R. (1965).** A Simplified Lead Citrate Stain for Use in Electron Microscopy. J. Cell. Biol. **25**: 407-408.

[18] **Schultz, J., Milpetz, F., Bork, P. and Ponting, C.P. (1998).** SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA **95**: 5857-5864.

[19] **Moller, S., Croning, M.D. and Apweiler, R. (2001).** Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics **17**: 646-653.

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

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

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

[23] **Gutsche, J., Remminghorst, U. and Rehm, B.H.A. (2005).** Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie **88**: 245-251.

[24] **Stover, C.K. et al. (2000).** Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406**: 959-964.

[25] **Käll, L., Krogh, A. and Sonnhammer, E.L. (2005).** An HMM posterior decoder for sequence feature prediction that includes homology information. Bioinformatics **21** (Suppl 1): i251-i257.

[26] **Nagata, Y., Futamura, A., Miyauchi, K. and Takagi, M. (1999).** Two different types of dehalogenases, LinA and LinB, involved in gamma-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26 are localized in the periplasmic space without molecular processing. J. Bacteriol. **181**: 5409-5413.

[27] **Ginalski, K., Elofsson, A., Fischer, D. and Rychlewski, L. (2003).** 3D-Jury: a simple approach to improve protein structure predictions. Bioinformatics **19**: 1015-1018.

[28] **Akama, H., Matsuura, T., Kashiwagi, S., Yoneyama, H., Narita, S., Tsukihara, T., Nakagawa, A. and Nakae, T. (2004).** Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. J. Biol. Chem. **279**: 25939-25942.

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

[30] **Bateman, A. et al. (2004).** The Pfam protein families database. Nucleic Acids Res. **32**: D138-D141.

[31] **Ohman, D.E. and Chakrabarty, A.M. (1981).** Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate. Infect. Immun. **33**: 142-148.

[32] **Holloway, B.W., Matsumoto, H. and Phibbs Jr., P.V. (1986).** The chromosome map of *Pseudomonas aeruginosa* PAO. Acta Microbiol. Pol. **35**: 161-164.

[33] **Mathee, K. et al. (1999).** Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiol. **145**: 1349-1357.

[34] **Simon, R., Priefer, U. and Pühler, A. (1983).** A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio-Technology **1**: 784-791.

[35] **Peters, J.E., Thate, T.E. and Craig, N.L. (2003).** Definition of the *Escherichia coli* MC4100 genome by use of a DNA array. J. Bacteriol. **185**: 2017-2021.

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

Chapter IV

Membrane topology and site-specific mutagenesis of Alg8, a putative glycosyltransferase involved in alginate polymerisation by *Pseudomonas aeruginosa*

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Abstract

Alg8 has been proposed to be the catalytic subunit of the alginate polymerase of *Pseudomonas aeruginosa*. Alg8 shows an amino acid sequence similarity of about 19.6 % to SpsA, a processive glycosyltransferase, and is localized in the cytoplasmic membrane. The membrane topology of Alg8 was determined by a combination of topology prediction algorithms and construction of C-terminal reporter enzyme fusions. Analysis of seven PhoA and LacZ fusions, respectively, confirmed the prediction that Alg8 contains one transmembrane (TM) domain at its N terminus and 3 TM domains at its C terminus. The extended loop between TM 1 and 2 is entirely located in the cytoplasm as indicated by fusion protein analysis. The C terminus (41 amino acid residues) encompassing TM 4 is essential for Alg8 function, which was indicated by fusion protein analysis. Site-directed mutagenesis of 11 conserved residues of Alg8 in the cytosolic loop region was used to elucidate the involvement of several conserved amino acids in Alg8 function. Replacement of the highly conserved Asp-191, Asp-193, Asp-298, Asp-299 and Lys-300 by alanine, respectively, resulted in abolishment of in *vivo* activity. Asp-191 and Asp-193 (DXD motif) might be involved in GDP-mannuronic acid binding, whereas Asp-298 and Asp-299 are candidates for the acid-base catalyst. Replacement of Asp-182, Asp-184, Cys-247, Arg-266, Asp-282, and Glu-285 did not abolish *in vivo* activity of Alg8, which suggested no direct involvement in the polymerisation reaction. Our model for Alg8 membrane topology suggests a large cytosolic domain, which contains aspartic acid residues involved in substrate binding and polymerisation.

Introduction

Alginates are linear exopolysaccharides consisting of β-1,4-linked β-d-mannuronic acid and its C5 epimer α-l-guluronic acid (29). The biosynthesis of alginate is a common feature in many brown seaweeds but only the two bacterial genera *Pseudomonas* and *Azotobacter* are known to produce alginates (18, 25). Although both genera produce alginate as an extracellular matrix material during their vegetative growth phase, its monomer distribution, polymer properties and biological function are quite different. While *Azotobacter vinelandii* synthesizes the alginate matrix to form two capsule-like layers required for maintaining structural integrity during a differentiation process leading to a desiccation resistant cyst (24), the biological function of alginate produced by *Pseudomonas aeruginosa* is quite different. *P. aeruginosa* is a well characterized opportunistic human pathogen and its alginate production is reported to be an important virulence factor during the infectious process of human epithelia (9). Infections of the respiratory tract are the major contributing factor causing high morbidity and mortality in cystic fibrosis patients (17). The production of extensive quantities of alginate results in the development of a characteristic mucoid phenotype and allows the formation of differentiated biofilms which restrict diffusion of clinical antibiotics and protect embedded cells against human antibacterial defence mechanisms (19).

Although alginate is primarily synthesized as polymannuronate from its cytosolic precursor GDP-mannuronic acid (29), it can undergo modification in form of acetylation or epimerization while traversing the periplasm (37), before being exported through the alginate outer membrane channel AlgE (26, 27). The acetylation occurs at position O2 and/or O3 of mannuronic acid residues and involves the proteins AlgIJF which presumably form a complex that transports a so far unidentified cytosolic acetyl donor across the cytoplasmic membrane and subsequently catalyzes the periplasmic acetylation reaction (8). Epimerization of unacetylated mannuronic acid residues is catalyzed by the C5-mannuronan-epimerase AlgG (7), which is proposed to have not only epimerase but also structural function, putatively contributing to an alginate protecting, aligning and guiding scaffold that protects the nascent alginate chain against degradation by the periplasmic alginate lyase AlgL (10, 12). The role of the alginate lyase is not fully elucidated yet, but recent publications showed the requirement for alginate production and suggested its role in clearing the periplasm of misfolded alginate not exported to the extracellular environment (1, 14). The two periplasmic proteins AlgK and AlgX are reported to contribute to the alginate scaffold structure, as indicated by the secretion of free uronic acid monomers or oligomers into culture supernatants by their respective deletion mutants (13, 31). Although being reported as part of the scaffold, AlgX might exert its function via interaction with the periplasmic

serine protease homologue MucD (11). Alg44 was recently shown to be localized to the periplasm and is proposed to be involved in posttranslational regulation of alginate biosynthesis via interaction with the bacterial second messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (28). Besides its regulatory function it is proposed to be a membrane fusion protein which might bridge the periplasm and facilitates contact between the cytoplasmic membrane protein Alg8 and the alginate outer membrane channel AlgE (28).

The proposed catalytic subunit of the alginate polymerase is Alg8, which was shown to be localized in the cytoplasmic membrane and is essential for alginate polymerisation (30). Evidence was recently provided using an *in vitro* alginate polymerisation assay that the alginate polymerase is a multiprotein complex constituted by proteins localized in the cytoplasmic membrane, outer membrane and the periplasm. The key role of Alg8 in alginate polymerisation was further emphasized by the finding that additional copy numbers of Alg8 lead to a strong overproduction of alginate (30). Additionally, the isogenic *alg8* deletion mutant did not secrete uronic acids, which were thought to be derived from degradation of misguided and unprotected alginate. This again suggested a direct involvement of Alg8 in alginate polymerisation. Uronic acids were released in the *algK*, *algX* and *algG* (epimerase gene) deletion mutants, respectively, which suggested a role of the respective proteins to guide and protect the nascent alginate chain. Hydrophobic cluster analysis of Alg8 indicated similarities to processive β-glycosyltransferases (GT) (e.g. cellulose or chitin synthase) (34), which are generally transmembrane proteins that synthesize polysaccharide or oligosaccharide chains by transferring the sugar residue from an activated donor substrate to a growing acceptor molecule. These different proteins are related based on their sequence pattern and especially with respect to the presence of conserved motifs and catalytic residues (33). The catalysis is believed to involve a general base, which assists in the deprotonation process of the nucleophilic hydroxyl of the acceptor, and an oxocarbenium-ion-like transition state similar to that proposed for glycosidases (39). A threading model of Alg8 was developed based on the crystal structure of SpsA, a glycosyltransferase involved in spore coat formation of *Bacillus subtilis* (5, 30).

In the present study we analyzed the predicted membrane topology of protein Alg8 by combining different topology prediction algorithms with experimentally obtained data using reporter gene fusions employing the alkaline phosphatase (PhoA) and the β-galactosidase (LacZ). Furthermore, the structural model of Alg8 and its alignment with related GTs was analyzed to identify possible catalytic residues or highly conserved structural motifs. These residues/motifs were targeted by site-directed mutagenesis and the protein function and activity was monitored assaying the *in vivo* alginate polymerisation activity. Since limited structural or crystallographic data are available about processive GTs, and given the large sequence diversity of the GT family, the

identification of catalytic residues in Alg8 might provide new insights into the catalysis of processive GTs. This is of particular interest because Alg8 represents a unique example for a glycosyltransferase involved in the synthesis of an exopolymer solely composed of uronic acid residues.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. All *E. coli* strains were grown in LB medium at 37 °C and *E. coli* S17-1 (36) was used for conjugative transfer of mob site containing pBBR1MCS-5 (16) derivatives. When required, antibiotics were added to the media using the following concentrations: ampicillin, 100 μg/ml; gentamicin, 10 μg/ml. *P. aeruginosa* PDO300 (21) and PDO300Δalg8 (30) were used in the present study and were cultivated in LB or PI(A) medium (Pseudomonas isolation [agar] medium: 20 g of peptone, 10 g of K_2SO_4, 1.4 g $MgCl_2$, 0.025 g of Triclosan, and 20 ml of glycerol per liter) at 37°C and, if required, gentamycin was added at a concentration of 100 μg/ml. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo.).

Isolation, analysis and manipulation of DNA. General cloning procedures were performed as described previously (32). The HPLC-purified primers used in the mutagensis study were purchased from Sigma Genosys, deoxynucleoside triphosphate, *Taq* and Platinum *Pfx* polymerases were purchased from Invitrogen. DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic sequencer.

Site-directed mutagenesis of Alg8. For the generation of the 11 site-directed mutants of Alg8 the native *alg8* gene of plasmid pBBR1MCS-5:alg8 (30) was excised using restriction endonucleases *Nde*I and *Pst*I and ligated into *Nde*I-*Pst*I hydrolyzed cloning vector pGEM-T_{Easy} (Promega) which resulted in a plasmid with smaller size and higher copy number. The resulting plasmid pGEM:alg8 was used as template for the PCR driven site-directed mutagenesis using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene). The DNA template concentration was adjusted to 35 ng dsDNA and each primer pair (direct and complement) (Table 1) was used at concentrations described in the manufacturer's manual. After confirmation of the mutagenesis by DNA sequencing, the mutated *alg8* gene was excised by *Nde*I and *Pst*I, and the resulting fragment was used to replace the native *alg8* containing *Nde*I and *Pst*I fragment of plasmid pBBR1MCS-5:alg8.

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

Strains, plasmids or oligonucleotides	Description	Source or reference
Strains		
P. aeruginosa		
PDO300	Δ*mucA22* variant of PAO1; Alg^+	(21)
PDO300Δalg8	Δ*alg8*; Alg^-	(30)
E. coli		
TOP10	*E. coli* cloning strain	Invitrogen
S17-1	*thi*-1; *proA*, *hsd*R*17* (r_k^-, m_k^+), *recA1*, *tra*-gene of plasmid RP4 integrated in chromosome	(36)
Plasmids		
pBBR1MCS-5	Gm^r; Broad-host-range vector; $P_{(lac)}$	(16)
pBBR1MCS-5:alg8	*Hin*dIII-*Bam*HI fragment comprising gene *alg8* inserted into vector pBBR1MCS-5	(30)
pBBR1MCS-5:alg8lacZ	Translational Alg8-LacZ fusion, inserted into vector pBBR1MCS-5	(30)
pBBR1MCS-5:alg8phoA	Translational Alg8-PhoA fusion, inserted into vector pBBR1MCS-5	(30)
pBBR1MCS-5:alg8N-90-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 90 amino acids of Alg8	This study
pBBR1MCS-5:alg8N-124-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 124 amino acids of Alg8	This study
pBBR1MCS-5:alg8N-184-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 184 amino acids of Alg8	This study
pBBR1MCS-5:alg8N-256-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 256 amino acids of Alg8	This study
pBBR1MCS-5:alg8N-363-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 363 amino acids of Alg8	This study
pBBR1MCS-5:alg8N-424-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 424 amino acids of Alg8	This study
pBBR1MCS-5:alg8N-456-lacZ / phoA	Translational Alg8-LacZ or PhoA fusion, containing first 456 amino acids of Alg8	This study
Oligonucleotides		
alg8N(HiSDNd)	CCGGGAAGCTTGAGGAGCACAGCCATATGGAACTGATGATGGAAACTTACAAA	
Alg8N-90(Ba)	ATAATAGGATCCGCCGGGTCGGCCGCGCTGCCCAACTG	
Alg8N-124(Ba)	AAGATAGGATCCGCCGGGTAGCCGCTGTCGATGGCTTCG	
Alg8N-184(Ba)	AAGATAGGATCCGCGTCGTCGTCCGGCAGGTGCCGGGAAATCGC	
Alg8N-256(Ba)	CATAGAGGATCCGCCAGCACGCGCTTGGACAGGGCCATCG	
Alg8N-363(Ba)	GATTTAGGATCCGCGCCCAGGCGCCGCGCGCCGAGCTTGAGC	
Alg8N-424(Ba)	ATATATGGATCCGCGATGCGGTGCCCGGAGAGCGAGAGGAGC	
Alg8N-456(Ba)	ATAATAGGATCCGCGGTCCAGGACTGCCGGTCGAGGC	
D182Adirect	CCCGGCACCTGCCGGCCGACGACGCGGTGGTC	
D182Acomplement	GACCACCGCGTCGTCGGCCGGCAGGTGCCGGG	
D184Adirect	GCACCTGCCGGACGACGCCGCGGTGGTCGCGG	
D184Acomplement	CCGCGACCACCGCGGCGTCGTCCGGCAGGTGC	
D191Adirect	GGTGGTCGCGGTGATCGCCGGCGACACCGTGCTCG	
D191Acomplement	CGAGCACGGTGTCGCCGGCGATCACCGCGACCACC	
D193Adirect	GGTGATCGACGGCGCTACCGTGCTCGACC	
D193Acomplement	GGTCGAGCACGGTAGCGCCGTCGATCACC	

C247Adirect	CCACATCAACATGGCCTCGATGGCCCTGTCC
C247Acomplement	GGACAGGGCCATCGAGGCCATGTTGATGTGG

Table 1: (Cont) Bacterial strains, plasmids and oligonucleotides used in this study

Strains, plasmids or oligonucleotides	Description	Source or reference
Oligonucleotides		
R266Adirect	GATGTCGGTGTTCGCCGCCCGGGTGGTGACC	
R266Acomplement	GGTCACCACCCGGGCGGCGAACACCGACATC	
D282Adirect	CGACGTCGAGAACGCCCACCTGGAGCACTGG	
D282Acomplement	CCAGTGCTCCAGGTGGGCGTTCTCGACGTCG	
E285Adirect	CGAGAACGACCACCTGGCGCACTGGCGCCTGGG	
E285Acomplement	CCCAGGCGCCAGTGCGCCAGGTGGTCGTTCTCG	
D298Adirect	GTTCCTCACCGGCGCCGACAAGTCCAGCTGG	
D298Acomplement	CCAGCTGGACTTGTCGGCGCCGGTGAGGAAC	
D299Adirect	CCTCACCGGCGACGCCAAGTCCAGCTGGTTCAGC	
D299Acomplement	GCTGAACCAGCTGGACTTGGCGTCGCCGGTGAGG	
K300Adirect	CCTCACCGGCGACGACGCGTCCAGCTGGTTCAGC	
K300Acomplement	GCTGAACCAGCTGGACGCGTCGTCGCCGGTGAGG	

Construction of reporter gene plasmids and fusion proteins. For the generation of the different fusion constructs 7 derivatives of gene *alg8* were amplified by PCR using the 5'-end primer alg8N(HiSDNd) and one of the 7 3'-end fusion primers (Table 1). The PCR products were hydrolyzed with *Nde*I and *Bam*HI and were used to replace the *Nde*I-*Bam*HI fragment containing the native *alg8* gene in plasmids pBBR1MCS-5:alg8phoA (30) and pBBR1MCS-5:alg8lacZ (30), respectively, in order to generate translational PhoA and LacZ fusions.

Alkaline phosphatase and β-galactosidase activity assays. Alkaline phosphatase and β-galactosidase enzymatic assays were performed according to the methods of Miller (22) and Manoil (20), respectively. Cells of *P. aeruginosa* were grown overnight in LB with the appropriate antibiotic, and the cultures were diluted 1:50 in LB medium. The cells were allowed to grow until the cultures reached an optical cell density at 600 nm of 0.4 to 0.6. The enzyme assays were performed in 1 ml of these cultures, and 1 ml was used for freeze-drying to determine the cellular dry weight. PhoA activity was determined by the rate of *p*-nitrophenylphosphate hydrolysis, taking the amount of enzyme hydrolyzing 1 μmol of substrate per 1 min at 37 °C as a unit of enzymatic activity. The results are given as average values of at least four independent experiments.

***In vivo* alginate polymerase activity.** The relative *in vivo* alginate polymerase activity was assessed by quantification of the alginate produced based on the expression of *alg8* gene and its derivatives in and *alg8*-negative isogenic mutant. A total of 2 ml of bacterial overnight cultures

was harvested (4000 x *g*, 15 min) at 4 °C and washed twice with saline. Then, 200 µl of cell suspension was plated onto PIA medium and incubated 72 h at 37 °C. Cells of two agar plates were scraped off by using a sterile spatula and washed twice with 40 ml of saline. When viscosity of the solution was too high for separation of cells, saline was added to a total volume of 300 ml in order to allow sedimentation of cells during centrifugation (6000 x *g*, 45 min). Cellular sediments were freeze-dried, and the final weight was determined. Alginate supernatants were precipitated with 1 vol of ice-cold isopropanol, and raw-alginate was harvested and freeze-dried. For further purification, the precipitated alginate was redissolved in buffer containing 0.05 M Tris-HCl and 10 mM $MgCl_2$ (pH 7.4) to a final concentration of 0.5% (w/v), followed by incubation with 15 µg of DNase I/ml and 15 µg of RNase A/ml at 37 °C for 6 h. Pronase E was added to a final concentration of 20 µg/ml, and the solution was incubated for further 18 h at 37 °C. Alginate solutions were dialyzed against 10 liters of ultrapure H_2O for 72 h. Subsequently, the alginate was precipitated with 1 volume of ice-cold isopropanol and freeze-dried for quantification and uronic acid analysis.

Uronic acid assays. Alginate concentrations were assayed by a modification of the Blumenkrantz and Asboe-Hansen protocol (4), using purified *P. aeruginosa* PDO300 alginate (100% [w/w] uronic acid content) as a standard. Briefly, purified alginate samples were dissolved in ultrapure H_2O to give 1 mg/ml alginate stock solutions, whereby concentrations between 0.25 and 0.05 mg/ml were used in the colorimetric assay. Then, 200 µl of the 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. The mixture was incubated at 100 °C for 5 min and then cooled down on ice for further 5 min. Finally, 20 µl of *m*-hydroxybiphenyl reagent (0.15% *m*-hydroxybiphenyl in 0.125 M NaOH) was added, and the reaction was mixed for 1 min. For each sample or dilution a negative control was set up using 0.0125 M NaOH instead of the hydroxybiphenyl reagent. The uronic acid concentrations were determined spectrophotometrically at a wavelength of 520 nm.

Results and Discussion

Membrane topology of Alg8. In a previous study the topology of Alg8 was analyzed by constrained topology prediction using only one experimentally confirmed localization (30). Alkaline phosphatase activity was detected when PhoA was fused to the C terminus of Alg8, which demonstrated a periplasmic localization of the C terminus (30). To verify the predicted membrane topology, 14 reporter gene fusions were constructed. To determine protein fusion

points, the membrane topology and the secondary structure of Alg8 was assessed using different predictions and databases like SMART (35), TMHMM2 (23), Phobius (15) and jPred (6). Since different predictions varied with respect to the localization of the last three TM regions the C-terminal region of Alg8 was particularly targeted by fusion protein analysis (Fig. 1).

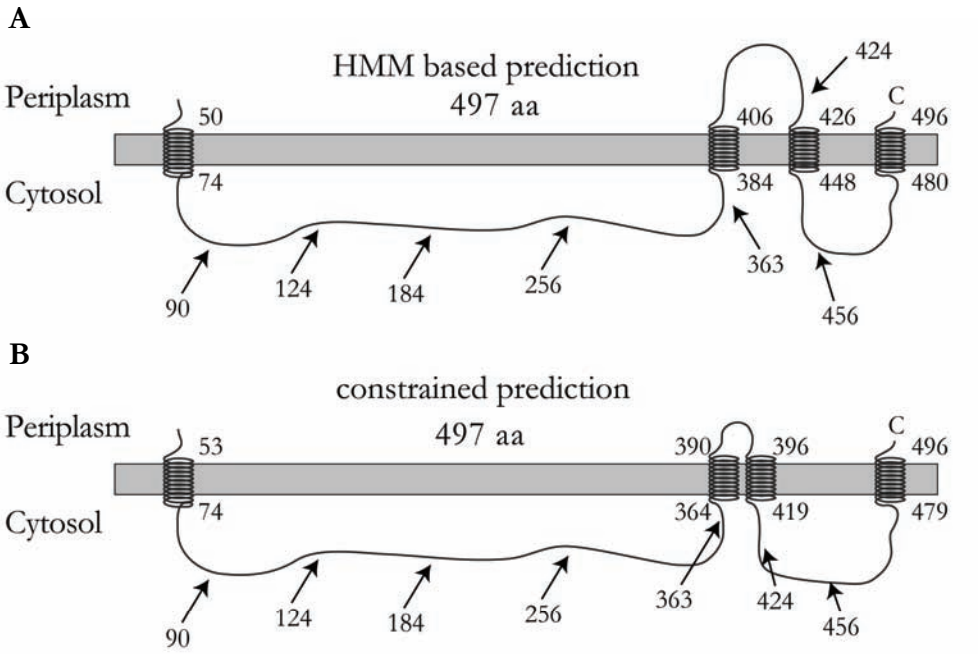

Figure 1: **Predicted membrane topology of Alg8. The HMM based prediction was generated by SMART (A), the constrained prediction was performed by using Phobius (B). All experimentally analyzed locations were used for the constrained prediction. The primary structure of Alg8 after removal of the potential N-terminal signal sequence was considered.**

In order to not disrupt any extended helical secondary structure motif which might form transmembrane segments, the secondary structure of the C terminus was analyzed with jPred, which combines the results of 6 different prediction algorithms, and fusion points were localized outside of predicted helical regions to allow proper folding (Fig. 2).

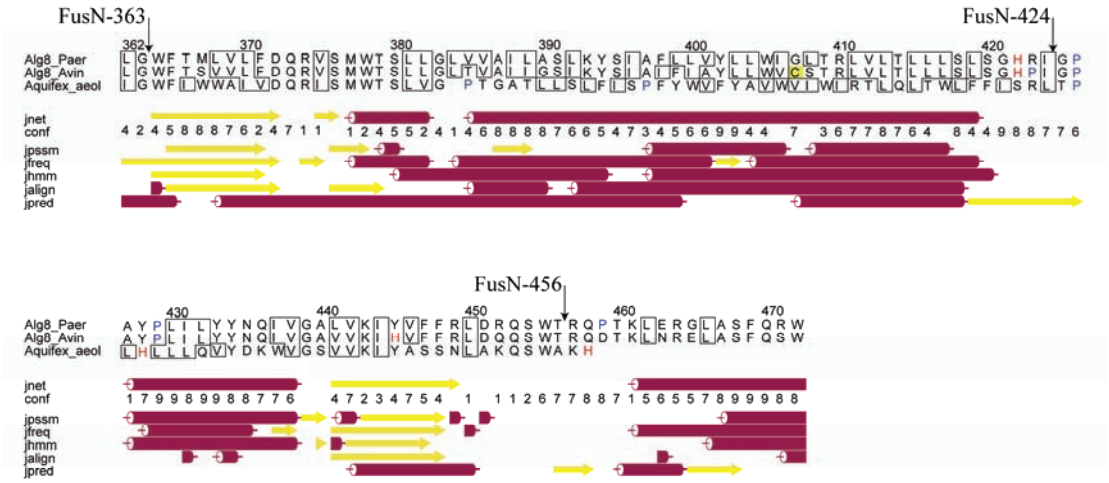

Figure 2: **Secondary structure prediction of Alg8 using program jPred. The structure was predicted using several different algorithms whereby helical stretches are illustrated as pipes and**

areas comprising β-strands as arrows. The black vertical arrows indicate the positions that were used as fusion points for the generation of the reporter protein fusions.

The extended helical region between amino acids 377 and 420 was previously predicted to contain only one transmembrane segment (30) (Fig. 1A), but jPred predictions indicated that it might comprise 2 transmembrane segments separated by only a short loop region (Fig. 2).

To analyze the membrane topology, fusions to PhoA and LacZ were generated after amino acids Gly-363 (FusN-363), Ile-424 (FusN-424), and Trp-456 (FusN-456), respectively. Strong β-galactosidase activity (> 65 U/mg CDW) of the respective LacZ-fusion proteins was determined and the corresponding alkaline phosphatase activity was only very low (Table 2), which imposed a cytosolic localization of all three fusion positions. To confirm the cytosolic localization of the large cytosolic loop that comprises the similarities to the GTs and for which a threading model was developed, 4 fusions to PhoA and LacZ, respectively, were made after residues Pro-90 (FusN-90), Pro-124 (FusN-124), Asp-184 (FusN-184), and Leu-256 (FusN-256), whereby all of these residues were located at the beginning or end of short predicted β-strands. For all fusion points high LacZ activity could be detected whereas PhoA activity was very low (Table 2). The use of all experimentally confirmed fusion point locations as well as the established periplasmic localization of the C terminus for constrained topology prediction suggested that the topology of Alg8 comprises at least 4 transmembrane helices. The data indicated a TM domain between residues 53 and 74, a large cytosolic loop between 74 and 364, and three TM domains spanning residues 364-390, 396-419, and 480-496 (Fig. 1B). The 290 amino acid residues comprising cytosolic loop region represents the enzymatic domain found in GTs.

Table 2: Enzymatic activity of Alg8-LacZ and Alg8-PhoA hybrids as well as the respective *in vivo* alginate polymerase activity. The values illustrated represent the mean of at least 3 independent measurements ± standard deviation.

Fusion position	LacZ activity [U/mg]	PhoA activity [U/mg]	*In vivo* alginate polymerase activity [%]
FusN-90	56.131 ± 3.244	0.404 ± 0.153	0
FusN-104	53.909 ± 10.460	0.386 ± 0.025	0
FusN-184	79.281 ± 5.456	0.350 ± 0.056	0
FusN-256	71.422 ± 1.182	0.346 ± 0.122	0
FusN-363	69.947 ± 2.263	0.382 ± 0.041	0
FusN-424	66.337 ± 2.540	0.505 ± 0.010	0
FusN-456	98.163 ± 16.168	0.407 ± 0.025	0
Alg8 C-terminal fusion	2.258 ± 0.629	7.664 ± 1.091	100
pBBR1MCS-5	0.836 ± 0.578	0.059 ± 0.027	0

The probability value for the existence of a signal sequence is 0.48 with a predicted cleavage site after residue 31 (SignalP 3.0 (3)), which neither confirms nor negates a signal

sequence. Although the existence of a signal sequence is not clear, predictions indicate that it will not lead to a different membrane topology, since the hydrophobic signal sequence is predicted to contain a transmembrane domain, which would cross the cytoplasmic membrane into the periplasm. None of the 14 constructs restored the alginate negative phenotype of strain *P. aeruginosa* PDO300Δalg8 (data not shown), which indicates the requirement of the C terminus of Alg8 for alginate production. Interestingly, the deletion of the C-terminal 41 amino acid residues encompassing TM 4 did already abolish *in vivo* Alg8 activity. Thus the C terminus is required for Alg8 functions, which has been also shown for the chitin synthase (NodC) (2).

Site-directed mutagenesis of Alg8. To analyze the involvement of previously suggested amino acid residues in the catalytic function of Alg8 (30) and to investigate the suitability and accuracy of our protein model, we generated 11 site-directed mutants of Alg8 (Fig. 3). The structural model indicated that the residues Asp-193, Cys-247, and Asp-282 are located in or adjacent to the core structure of the enzymatic domain (74-364) (30). Since these and other homologous residues were reported to be responsible for the nucleotide-sugar binding and catalysis in SpsA (5), these amino acids have been replaced by alanine, and the *in vivo* protein function of Alg8 was monitored by quantification of alginate production.

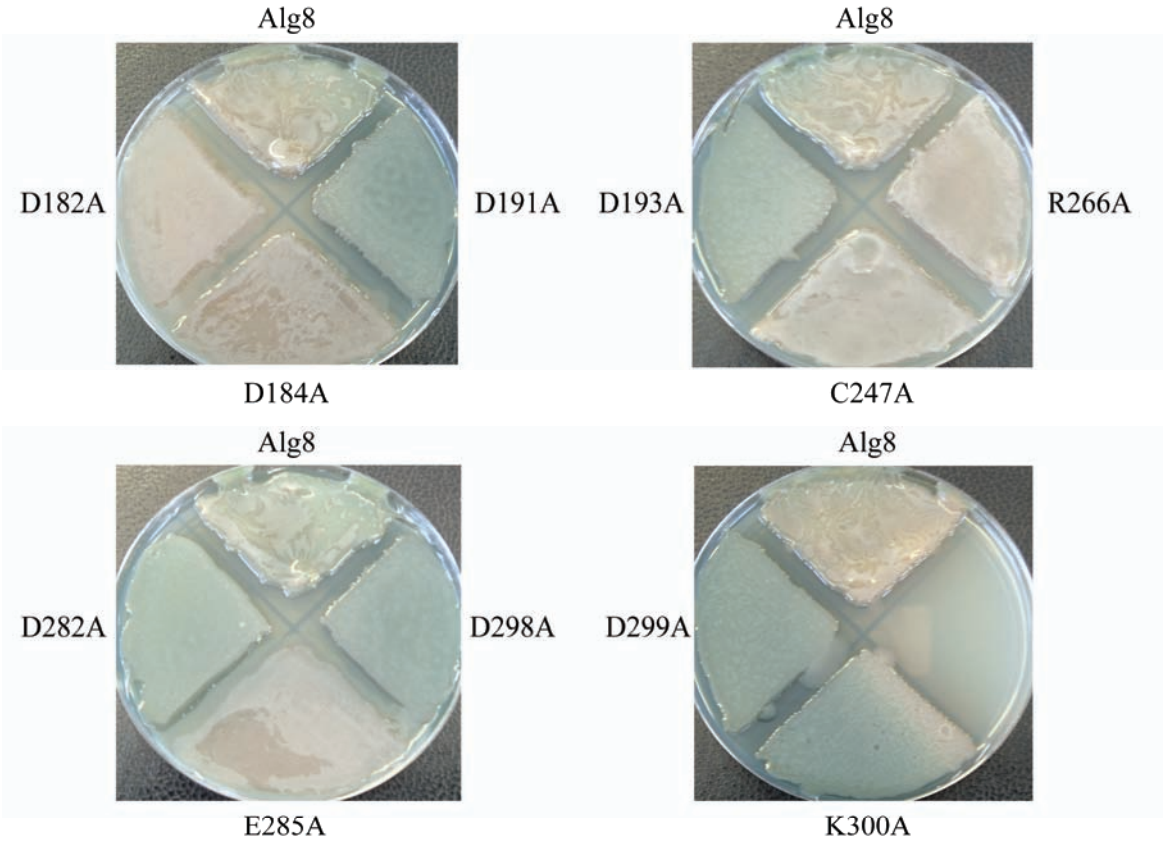

Figure 3: **Alginate production as indicated by mucoid phenotypes of *P. aeruginosa* PDO300Δalg8 harbouring various plasmids encoding Alg8 and its site-directed mutants. Cultivation was performed on PIA medium for 72 h at 37 °C.**

Glycosyltransferases catalyze the transfer of a sugar residue from an activated donor substrate to a growing acceptor molecule, hence a common feature of many of the different GT families is the so called DXD motif which is supposed to be involved in nucleotide-sugar binding (33, 39). Although this canonical motif contains two aspartic acid residues, the first (first position of the motif) is relatively variable while the second (third position of the motif) is quite well conserved amongst different GTs, but the motif itself can also be reduced to only one aspartic acid residue as seen in SpsA (5). The DXD motif is reported to follow a stretch of at least 4 hydrophobic residues, which often form a strand that runs through the core structure of the domain (39). Alg8 contains two putative DXD motifs of which the first one at positions Asp-182 and Asp-184 is not N-terminally flanked by 4 hydrophobic residues and the structural model suggested a peripheral localization (Fig. 4A). The second one at positions Asp-191 and Asp-193 is flanked by 4 hydrophobic residues and is located in the core structure of the Alg8 model.

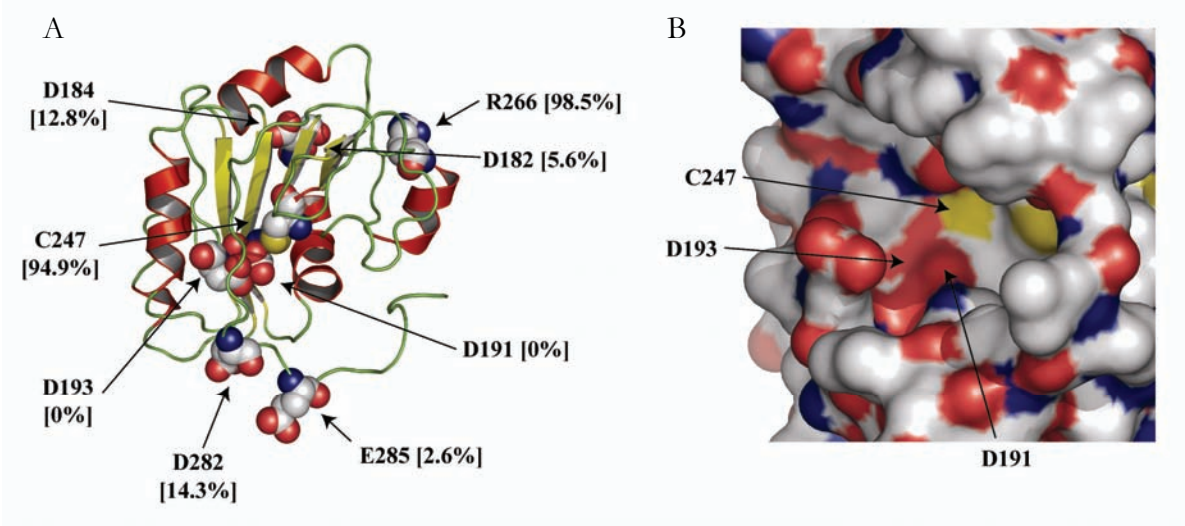

Figure 4: **Localization of the single amino acid residue replacements in the Alg8 model. Amino acid replacements were achieved by site-directed mutagenesis (see Table 3). Only the backbone of the model is shown, and the residues targeted for replacements are given as CPK format. The *in vivo* alginate production of the respective mutant in comparison to the native Alg8 is given in brackets.**

Earlier reports about the structure of processive GTs suggested the transfer of two sugar monomers at one catalytic step in order to prevent chain or enzyme rotation, given that the monomers in the growing chain are rotated by 180 degrees (34). This is reported to require two catalytic residues, but since many structures of GTs only show one catalytic base, the addition of one monomer at a time seems to be favoured. The experimental finding that not necessarily each DXD motif is involved in nucleotide sugar binding (LgtC contains 4 DXD motifs, yet only one is involved in ribose/manganese binding) is a further indication that the number of the motifs

within an ORF is not a reflection of the number of its catalytic sites (38). The replacement of the four aspartic acid residues in Alg8 by alanine showed that only the inactivation of the DXD motif at position 191 to 193 led to inactivation of *in vivo* alginate polymerase activity (Table 3). Whilst the mutagenesis of the first DXD motif decreased alginate production by about 90 %, alginate was still produced at detectable quantities and the decrease of alginate polymerase activity is concomitant with a slightly higher biomass production when compared to the overproducing mutants. Exchanging each of the aspartic acid residues at positions 191 or 193 led to complete abolishment of alginate polymerisation, suggesting that in contrast to other GTs, where the motif is degenerated or only one residues is involved (5, 38, 39), each of the two residues is involved in catalysis exerting a putative function in nucleotide uronic acid binding. A similar result was obtained for the α-1,3-mannosyltransferase Mnn1p, where inactivation of either of the two aspartates involved in the DXD motif led to complete elimination of enzyme activity without changing or interfering with protein folding (40).

Table 3: *In vivo* alginate polymerase activity as indicated by alginate and cellular dry mass production by *P. aeruginosa* PDO300Δalg8 strains harbouring plasmids encoding various site-directed Alg8 mutants

Strain	Mean ± SD		
	Alginate production (g/g CDM)	Cellular dry mass (CDM [g])	Relative *in vivo* alginate polymerase activity*
PDO300Δalg8 (pBBR1MCS-5)	ND[a]	0.168 ± 0.012	0
PDO300Δalg8 (pBBR1MCS-5:alg8)	1.782 ± 0.186	0.084 ± 0.005	100
PDO300Δalg8 (pBBR1MCS-5:alg8D182A)	0.101 ± 0.012	0.246 ± 0.006	5.6
PDO300Δalg8 (pBBR1MCS-5:alg8D184A)	0.227 ± 0.048	0.158 ± 0.014	12.8
PDO300Δalg8 (pBBR1MCS-5:alg8D191A)	ND	0.172 ± 0.040	0
PDO300Δalg8 (pBBR1MCS-5:alg8D193A)	ND	0.193 ± 0.045	0
PDO300Δalg8 (pBBR1MCS-5:alg8C247A)	1.690 ± 0.629	0.056 ± 0.019	94.9
PDO300Δalg8 (pBBR1MCS-5:alg8R266A)	1.754 ± 0.412	0.083 ± 0.025	98.5
PDO300Δalg8 (pBBR1MCS-5:alg8D282A)	0.259 ± 0.026	0.163 ± 0.012	14.3
PDO300Δalg8 (pBBR1MCS-5:alg8E285A)	0.048 ± 0.014	0.230 ± 0.026	2.6
PDO300Δalg8 (pBBR1MCS-5:alg8D298A)	ND	0.124 ± 0.007	0
PDO300Δalg8 (pBBR1MCS-5:alg8D299A)	ND	0.157 ± 0.008	0
PDO300Δalg8 (pBBR1MCS-5:alg8K300A)	ND	0.110 ± 0.001	0

[a] ND, not detectable; * Given in % of native Alg8 *in vivo* polymerase activity.

According to the structural model, Asp-191 and Asp-193 are located in a deep cleft within the core structure of the enzymatic domain (Fig. 4A and B), while the 4 preceding hydrophobic residues form a short β-strand that crosses the protein core and positions the motif into the protein core. In the threading model Asp-182 and Asp-184 are located outside the core structure and are surface exposed, and the decrease in *in vivo* alginate polymerase activity by replacement of either of these residues suggests no involvement in the catalytic reaction mechanisms. In some GTs the aspartic acid residues are shown to coordinate a divalent metal ion (mostly Mn^{2+}) that is

involved in coordination of the phosphates of the sugar-nucleotide. The mutational inactivation of the motif is reported to prevent nucleotide binding and therefore abolishing enzymatic function without altering the enzyme tertiary structure (40).

Besides the nucleotide sugar binding motif, all GTs contain a catalytic center, which in SpsA was initially suggested to be either a catalytic triad consisting of Asp-158, His-159 and Cys-160 or a single aspartic acid residue Asp-191 (5). None of the triad residues is reported to be specifically conserved amongst GTs, but according to our model Cys-247 corresponds to Cys-160 in the SpsA model and seems to be located in the protein core structure of Alg8. However, many authors reported lately that an aspartate or glutamate acted as Brønsted base and therefore as base catalyst in the active center of many different GT families (38, 39). To identify possible candidates of Brønsted bases and further conserved residues, which might be involved in alginate polymerisation, an alignment of Alg8 with the chitin synthase I from *Saccharomyces cerevisiae* (ChsI) and NodC, a chitin oligosaccharide synthase from *Mesorhizobium loti*, was created by using program ClustalW. The alignment alluded to the conserved residues Cys-247, Arg-266, Asp-282, Glu-285, Asp-298, Asp-299, and Lys-300, which were each replaced by alanine. *In vivo* alginate polymerase activity assays suggested that Cys-247 and Arg-266 are not involved in alginate polymerisation (Table 3). Replacement of Asp-282 and Glu-285, putative candidates for the acid-base catalyst in the active center, significantly decreased alginate polymerase activity to about 14.3 % and 2.6 % of wildtype Alg8 activity, respectively (Table 3). Since alginate polymerase activity was not abolished, these residues do not seem to be directly involved in the polymerisation reaction. Moreover, in the threading model these residues are located at the surface of the core structure (Fig 4A). Unfortunately, the structural model could not include the residues Asp-298, Asp-299 and Lys-300 because of weak similarities to the SpsA primary structure. However, the protein model indicated that those residues would be located in the cleft opposite to the DXD motif. Thus amino acid residues Asp-298, Asp-299, and Lys-300 could be involved in the catalytic reaction mechanism. The replacement of these residues resulted in a loss of alginate polymerase activity (Table 3). These findings suggested that this protein region could be involved in catalysing the polymerisation reaction with one of the aspartate residues presumably representing the Brønsted base. The second aspartate might be required to accommodate via metal ion coordination the additional carboxyl group found in the uronic acid moiety. Since replacements of all residues in this protein region abolished protein function, the acid-base catalyst function could not be mapped to single residue. The motif QXXRW, as described for many processive chitin and cellulose synthases, is suggested to be a common domain in processive enzymes which is proposed to hold the growing glucan chain in the active

site (33). Primary structure alignments of Alg8 with ChsI and NodC did not show a conserved QXXRW motif.

Alg8 has been proposed as the catalytic subunit of the alginate polymerase, because it shows similarities to GTs and is essential for alginate polymerisation. In this study, experimental evidence was provided that Alg8 is an integral cytoplasmic membrane protein with at least four TM domains. Although Alg8 shows primary structure similarities to other GTs, it has unique features such as the requirement of several aspartate residues and the lack of the QXXRW motif. These peculiarities found in Alg8 might reflect the nature of the unique substrate, GDP-mannuronic acid, as well as the unique product, alginate.

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References

1. **Bakkevig, K., H. Sletta, M. Gimmestad, R. Aune, H. Ertesvåg, K. Degnes, B. E. Christensen, T. E. Ellingsen, and S. Valla.** 2005. Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment. J. Bacteriol. **187:**8375-8384.
2. **Barny, M. A., E. Schoonejans, A. Economou, A. W. Johnston, and J. A. Downie.** 1996. The C-terminal domain of the *Rhizobium leguminosarum* chitin synthase NodC is important for function and determines the orientation of the N-terminal region in the inner membrane. Mol. Microbiol. **19:**443-453.
3. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:**783-795.
4. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484-489.
5. **Charnock, S. J., and G. J. Davies.** 1999. Structure of the nucleotide-diphospho-sugar transferase, SpsA from *Bacillus subtilis*, in native and nucleotide-complexed forms. Biochemistry **38:**6380-6385.
6. **Cuff, J. A., and G. J. Barton.** 2000. Application of multiple sequence alignment profiles to improve protein secondary structure prediction. Proteins **40:**502-511.

7. **Franklin, M. J., C. E. Chitnis, P. Gacesa, A. Sonesson, D. C. White, and D. E. Ohman.** 1994. *Pseudomonas aeruginosa* AlgG is a polymer level alginate C5-mannuronan epimerase. J. Bacteriol. **176:**1821-1830.
8. **Franklin, M. J., and D. E. Ohman.** 2002. Mutant analysis and cellular localization of the AlgI, AlgJ, and AlgF proteins required for O acetylation of alginate in *Pseudomonas aeruginosa*. J. Bacteriol. **184:**3000-3007.
9. **Gacesa, P., and N. J. Russell.** 1990. *Pseudomonas* infection and alginates : biochemistry, genetics, and pathology. Chapman and Hall, London ; New York
10. **Gimmestad, M., H. Sletta, H. Ertesvåg, K. Bakkevig, S. Jain, S.-J. Suh, G. Skjåk-Bræk, T. E. Ellingsen, D. E. Ohman, and S. Valla.** 2003. The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J. Bacteriol. **185:**3515-3523.
11. **Gutsche, J., U. Remminghorst, and B. H. A. Rehm.** 2006. Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex. Biochimie **88:**245-251.
12. **Jain, S., M. J. Franklin, H. Ertesvåg, S. Valla, and D. E. Ohman.** 2003. The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol. Microbiol. **47:**1123-1133.
13. **Jain, S., and D. E. Ohman.** 1998. Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J. Bacteriol. **180:**634-641.
14. **Jain, S., and D. E. Ohman.** 2005. Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infect. Immun. **73:**6429-6436.
15. **Käll, L., A. Krogh, and E. L. Sonnhammer.** 2004. A combined transmembrane topology and signal peptide prediction method. J. Mol. Biol. **338:**1027-1036.
16. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, 2nd, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175-176.
17. **Leid, J. G., C. J. Willson, M. E. Shirtliff, D. J. Hassett, M. R. Parsek, and A. K. Jeffers.** 2005. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-γ-mediated macrophage killing. J. Immunol. **175:**7512-7518.
18. **Linker, A., and R. S. Jones.** 1966. A new polysaccharide resembling alginic acid isolated from pseudomonads. J. Biol. Chem. **241:**3845-3851.
19. **Lyczak, J. B., C. L. Cannon, and G. B. Pier.** 2002. Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. **15:**194-222.

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

21. **Mathee, K., C. Sternberg, O. Ciofu, P. Jensen, J. Campbell, M. Givskov, D. E. Ohman, N. Hoiby, S. Molin, and A. Kharazmi.** 1999. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiol. **145:**1349-1357.

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

23. **Moller, S., M. D. Croning, and R. Apweiler.** 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics **17:**646-653.

24. **Moreno, S., R. Najera, J. Guzman, G. Soberon-Chavez, and G. Espin.** 1998. Role of alternative sigma factor *algU* in encystment of *Azotobacter vinelandii*. J. Bacteriol. **180:**2766-2769.

25. **Pindar, D. F., and C. Bucke.** 1975. The biosynthesis of alginic acid by *Azotobacter vinelandii*. Biochem. J. **152:**617-622.

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

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

28. **Remminghorst, U., and B. H. A. Rehm.** 2006. Alg44, a unique protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. FEBS Lett **580:**3883-3888.

29. **Remminghorst, U., and B. H. A. Rehm.** 2006. Bacterial alginates: from biosynthesis to applications. Biotechnol. Lett. **28:**1701-1712.

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

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

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

33. **Saxena, I. M., R. M. Brown, Jr., and T. Dandekar.** 2001. Structure-function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57:**1135-1148.

34. **Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia, and B. Henrissat.** 1995. Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. J. Bacteriol. **177:**1419-1424.

35. **Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting.** 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA **95:**5857-5864.

36. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vitro* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio-Technology **1:**784-791.

37. **Skjåk-Bræk, G., H. Grasdalen, and B. Larsen.** 1986. Monomer sequence and acteylation pattern in some bacterial alginates. Carbohydr. Res. **154:**239-250.

38. **Tarbouriech, N., S. J. Charnock, and G. J. Davies.** 2001. Three-dimensional structures of the Mn and Mg dTDP complexes of the family GT-2 glycosyltransferase SpsA: a comparison with related NDP-sugar glycosyltransferases. J. Mol. Biol. **314:**655-661.

39. **Ünligil, U. M., S. Zhou, S. Yuwaraj, M. Sarkar, H. Schachter, and J. M. Rini.** 2000. X-ray crystal structure of rabbit N-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily. EMBO J. **19:**5269-5280.

40. **Wiggins, C. A., and S. Munro.** 1998. Activity of the yeast MNN1 alpha-1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. Proc. Natl. Acad. Sci. USA **95:**7945-7950.

Chapter V

Attachment and biofilm architecture of a supermucoid *Pseudomonas aeruginosa*

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Abstract

The supermucoid *Pseudomonas aeruginosa* PDO300Δalg8 (pBBR1MCS-5:alg8) showed strongly impaired attachment and formed a thicker biofilm than the respective mucoid or nonmucoid strains. The supermucoid strain biofilm was characterized by large extended streamers. These results demonstrate that alginate impairs attachment and is required for formation of thick biofilms and large streamers.

Introduction

Alginate is an important virulence factor, and the conversion of the nonmucoid to the alginate-overproducing mucoid strains early after infection of cystic fibrosis patients is associated with a decline of pulmonary function and survival rate (7, 10). Alginate functions as extracellular matrix material enabling the formation of differentiated biofilms, in which diffusion of clinical antibiotics is decreased and the embedded cells are protected against human antibacterial defence mechanisms (5, 9). Although not required for *P. aeruginosa* biofilm formation (12), Nivens and colleagues (5) provided evidence that alginate plays a role in the formation of thick and three-dimensional biofilms. To further investigate the impact of alginate on attachment and biofilm architecture, we used a recently generated supermucoid strain, which showed an about 15-fold alginate overproduction, when compared to alginate producing mucoid *P. aeruginosa* (11).

Experiments

Quantitative analysis of attachment and biofilm formation. The attachment characteristics of the supermucoid strain PDO300Δ*alg8* (pBBR1MCS-5:*alg8*) were compared with wildtype strain PAO1, mucoid strain PDO300 and its alginate negative isogenic *alg8* deletion mutant (11). A modification of the solid surface assay (SSA) (6) was used to assess attachment and biofilm formation in microtiter plates 18 after incubation for 2, 4, 6, 24 h and 4 d. Stationary cultures at 37 ºC in Luria-Bertani 19 (LB) medium (containing gentamycin 300 µg/ml when appropriate), were adjusted to OD_{600} of 0.05 and 100 µl added to one column (8 wells) of each of five replicate 96-well tissue culture plates. After incubation at 37 ºC for the respective time, non-adherent bacteria were washed off by filling the wells 3 times with sterile water and then removing the well contents with gentle suction. Plates were then air dried and adherent bacteria stained with 100 µl 0.1% (w/v) crystal violet for 20 min at room temperature. The crystal violet was removed by washing as described above and dissolved in 100 µl (v/v) DMSO (dimethyl sulfoxide). After 20 min, the absorbance at 595 nm was measured. The data presented here are the average of 2 independent experiments each with 8 replicates. The results showed excellent intra-assay and inter-assay reproducibility, with minimal background. During the early attachment phase (2-4 hours) the nonmucoid strains (wild-type strain PAO1 and PDO300Δ*alg8*) showed significantly more attachment than any of the other strains (Fig. 1A). The supermucoid strain PDO300Δ*alg8* (pBBR1MCS-5:*alg8*) showed the weakest attachment at 2, 4 and 6 h, but complete biofilm formation after 4 days. Biofilm analysis after stringent (2) or gentle (see description above) washing was used to evaluate the strength of the attachment of the grown biofilm. The

difference between stringent and gentle washing was minimal in the PDO300Δ*alg8* mutant, where the extracellular matrix lacked alginate, while the greatest differences were observed in the alginate rich biofilms of PDO300 and of the supermucoid strain (Fig. 1B). These results suggest that alginate interferes with early attachment but subsequently promotes formation of a thick, although loosely attached, biofilm.

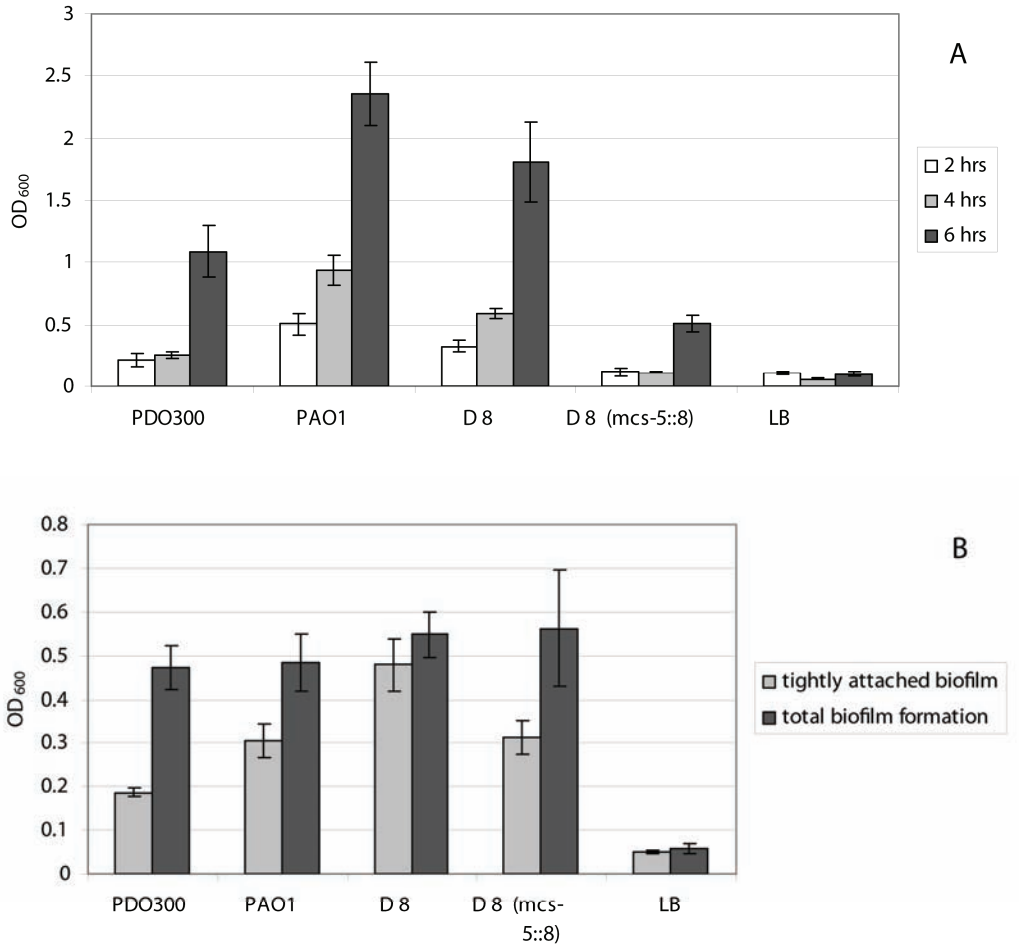

Figure 1: **SSA analysis of various *P. aeruginosa* strains. PDO300, *P. aeruginosa* PDO300; PAO1, *P. aeruginosa* PAO1; D8, *P. aeruginosa* PDO300Δ*alg8*; D8 (mcs-5:*8*), supermucoid strain; LB, uninoculated LB medium control. A, differences during early attachment phase; B, differences between loosely and tightly attached 4-days old biofilms (adherent biofilm after gentle or stringent washing respectively). Values and error bars represent the average and the standard deviation of 8 independent replicates, respectively**

Biofilm analysis. Biofilm morphology and architecture of strains PDO300, PDO300Δ*alg8*, and the supermucoid strain were analysed in a continuous culture flow-chamber as previously described. After 4 days of growth in Pseudomonas Isolation Medium (11), biofilms were stained with LIVE/DEAD BacLight bacterial viability kit (Molecular Probes Inc.) as previously described (1) and observed under a confocal laser scanning microscope (CSLM, LEICA TCS 4D)

using the appropriate filters. Strain PDO300 showed a mature fully differentiated biofilm, consisting of microcolonies and streamers (Fig. 2A), the biofilm's average thickness was 95±5 μm. Strain PDO300Δ*alg8* showed a thinner more uniform biofilm (65±5 μm) lacking streamers (2B). Interestingly, in this biofilm small clusters of cells were distinguishable, while both PDO300 and the supermucoid strain cells were embedded in the matrix and indistinguishable (Fig. 2A and 2D respectively). The control strain PDO300Δ*alg8* (pBBR1MCS-5) was analysed in parallel (Fig. 2C) and showed the same biofilm characteristics as PDO300Δ*alg8*. The supermucoid strain PDO300Δ*alg8* (pBBR1MCS-5:*alg8*) showed the thickest biofilm (115±5 μm). The biofilm appeared to be constituted mainly of very large streamers (Fig. 2D), reaching a thickness similar to those formed by PDO300. Proportion of dead and live cells did not differ among the tested strains (data not shown).

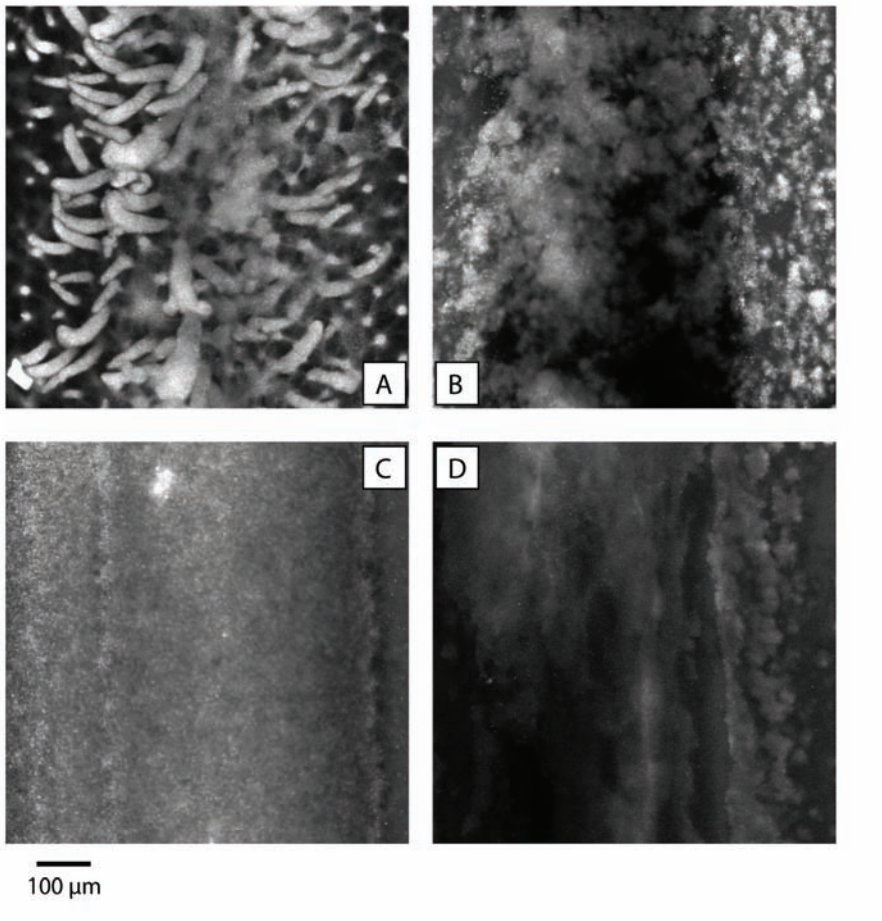

Figure 2: ***P. aeruginosa*** **biofilm formation in a continuous culture flow cell after 4 days from inoculation. A,** ***P. aeruginosa*** **PDO300; B,** ***P. aeruginosa*** **PDO300Δ*alg8*; C,** ***P. aeruginosa*** **PDO300Δ*alg8*** **(pBBR1MCS5); D,** ***P. aeruginosa*** **PDO300Δ*alg8*** **(pBBR1MCS-5:*alg8*). Magnification is 400x. The scale bar corresponds to 100 μm.**

Since alginate production by mucoid strains was suggested to reduce intracellular polyhydroxyalkanoate (PHA) synthesis and PHA synthesis was proposed to impact on biofilm

formation, we analysed the capability of PHA synthesis in the supermucoid strain (8) by GC-MS analysis, as previously described. The supermucoid strain PDO300Δ*alg8* (pBBR1MCS-5:*alg8*) showed an about 3-fold decreased PHA content when compared to the alginate-negative strain PDO300Δ*alg8* (pBBR1MCS-5) and an about 2-fold decreased PHA content when compared to the mucoid strain PDO300 (pBBR1MCS-5) (data not shown).

Discussion

In this study, the role of alginate in attachment and biofilm formation was studied using a recently genetically engineered supermucoid strain PDO300Δ*alg8* (pBBR1MCS-5:*alg8*), which produces 15-times more alginate, when grown as biofilm, than the alginate-producing mucoid strain PDO300. Exopolysaccharides produced by *P. aeruginosa* have been shown to be involved in attachment and biofilm formation (1, 3, 5, 6). Alginate has been shown to play a role in attachment to surfaces (4). However, the SSA analysis in this study clearly suggested that alginate overproduction impairs attachment to solid surfaces (Fig. 1A) as the mucoid strains showed the weakest attachment, whereas the alginate-negative strain and the nonmucoid wildtype showed the strongest attachment when assayed 2 to 6 h after inoculation (Fig. 1). In addition, a decreased intracellular PHA accumulation was found in the supermucoid strain suggesting that common metabolites are channelled into alginate biosynthesis as has been previously discussed (8). PHA negative mutants have been shown to be impaired in attachment (8). Nivens and colleagues (5) provided evidence that alginate contributes to the biofilm architecture. Since the supermucoid strain formed a thicker biofilm than the mucoid strain, alginate is presumably required for formation of biofilms exhibiting a certain thickness. The most striking difference found in the biofilm architecture of the supermucoid strain was the extensive formation of large streamers, which suggests a function of alginate in streamer formation (Fig. 2D).

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References

1. **Campisano, A., C. Schroeder, M. Schemionek, J. Overhage, and B. H. A. Rehm. 2006.** PslD is a secreted protein required for biofilm formation by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **72:** 3066-3068.
2. **Finelli, A., C. V. Gallant, K. Jarvi, and L. L. Burrows**. 2003. Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. J. Bacteriol. **185:** 2700-2710.
3. **Jackson, K. D., M. Starkey, S. Kremer, M. R. Parsek, and D. J. Wozniak. 2004.** Identification of *psl*, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. J. Bacteriol. **186:** 4466-4475.
4. **Mai, G. T., J. G. McCormack, W. K. Seow, G. B. Pier, L. A. Jackson, and Y. H. Thong. 1993.** Inhibition of adherence of mucoid *Pseudomonas aeruginosa* by alginase, specific monoclonal antibodies, and antibiotics. Infect. Immun. **61:** 4338-4343.
5. **Nivens, D. E., D. E. Ohman, J. Williams, and M. J. Franklin. 2001.** Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J. Bacteriol. **183:** 1047-1057.
6. **Overhage, J., M. Schemionek, J. S. Webb, and B. H. A. Rehm. 2005.** Expression of the *psl* operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation. Appl. Environ. Microbiol. **71:** 4407-4413.
7. **Pedersen, S. S., N. Hoiby, F. Espersen, and C. Koch. 1992.** Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax **47:** 6-13.
8. **Pham, T. H., J. S. Webb, and B. H. A. Rehm. 2004.** The role of polyhydroxyalkanoate biosynthesis by *Pseudomonas aeruginosa* in rhamnolipid and alginate production as well as stress tolerance and biofilm formation. Microbiology **150:** 3405-3413.
9. **Pier, G. B., F. Coleman, M. Grout, M. Franklin, and D. E. Ohman. 2001.** Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. Infect. Immun. **69:** 1895-1901.
10. **Rehm, B. H. A., E. Grabert, J. Hein, and U. K. Winkler. 1994.** Antibody response of rabbits and cystic fibrosis patients to an alginate-specific outer membrane protein of a mucoid strain of *Pseudomonas aeruginosa*. Microb. Pathog. **16:** 43-51.
11. **Remminghorst, U., and B. H. A. Rehm. 2006.** *In vitro* alginate polymerisation and the functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **72:** 298-305.

12. **Wozniak, D. J., T. J. Wyckoff, M. Starkey, R. Keyser, P. Azadi, G. A. O'Toole, and M. R. Parsek. 2003.** Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. Proc. Natl. Acad. Sci. U.S.A. **100:** 7907-7912.

Chapter VI

Biochemical analysis of alginate biosynthesis protein AlgX from *Pseudomonas aeruginosa*: purification of an AlgX-MucD (AlgY) protein complex

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Keywords: Alginate biosynthesis; AlgX; MucD (AlgY); Stress tolerance; Serine protease

Abbreviations: EPS, extracellular polymeric substance; FLP, *Saccharomyces cerevisiae* recombinase; *FRT*, Flp recombinase target; MALDI-TOF, matrix assisted laser desorption/ionisation time-of-flight; MS, mass spectrometry; PIA, *Pseudomonas* isolation agar

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Abstract

AlgX was found to be an essential protein for alginate biosynthesis, but its function is unknown. In this study, an isogenic, marker-free *algX*-knock out mutant was generated. In-frame fusions of *algX* with *phoA* and *lacZ* were analysed, respectively. No LacZ-activity was detected, but the PhoA fusion showed alkaline phosphatase activity. These data indicated that the C terminus of AlgX is located in the periplasm, but is not required for protein function. Accordingly, AlgX with C-terminal fusion of strep-tag II restored alginate production in the *algX*-negative mutant and was purified under native conditions from periplasmic and crude cell extracts, respectively. AlgX was identified by MALDI-TOF/MS analysis of tryptic peptides. TritonX-100 mediated solubilization of cytoplasmic membrane and subsequent strep-tag II affinity chromatography led to purification of an AlgX-MucD (AlgY) protein complex as identified by MALDI-TOF/MS analysis. These data suggested a protein-protein interaction between AlgX and MucD (AlgY) with a 1:1 stoichiometry. Thus AlgX might exert its function via interaction with MucD (AlgY). Immunoelectron microscopic localization of AlgX-strep-tag II suggested a localization close to the cytoplasmic membrane.

Introduction

Alginate is an exopolysaccharide, which is produced by brown algae and by the two bacterial genera *Pseudomonas* and *Azotobacter*, consisting of variable amounts of (1,4)-linked β-D-mannuronate and its epimer α-L-guluronate [1]. Bacterial alginate functions as extracellular matrix in biofilm formation, which plays an important role during infection by *Pseudomonas aeruginosa* [1]. Alginates are also considered as valuable biomaterial for various medical applications [1]. The *algX* gene from *P. aeruginosa* is part of the alginate biosynthesis gene cluster [2]. The function of AlgX is unknown and no orthologous proteins are found in the current databases. The *mucD* (*algY*) gene is located in the gene cluster *algU* (*algT*) *–mucABCD* (*algSNMY*) involved in transcriptional regulation of alginate biosynthesis genes [1]. MucD (AlgY) shows homologies to HtrA (DegP), a periplasmic serine protease involved in proteolysis of damaged proteins and necessary for heat resistance in *Escherichia coli* as well as being required for resistance to oxidative stress in *Salmonella typhimurium* [3,4]. MucD (AlgY) acts as negative regulator of alginate production. *MucD* (*algY*) mutants showed enhanced induction of *algD* transcription. The *algD* gene encodes the GDP-mannose dehydrogenase, a key enzyme of alginate biosynthesis. Moreover, MucD (AlgY) seems to play a role in stress resistance in *P. aeruginosa* as the inactivation of *mucD* (*algY*) results in a decreased resistance to H_2O_2 and heat killing [5]. It has been suggested that AlgX is localized in the periplasm contributing to a protein scaffold surrounding the nascent polymer from degradation by the alginate lyase AlgL [2, 6]. In this study, we generated the first isogenic, marker-free *algX* knock-out mutant in order to investigate the role of AlgX in alginate biosynthesis. We purified AlgX via the Strep-tag II/ Strep-Tactin-System and co-purified MucD (AlgY), indicating an interaction between these two proteins. The role of AlgX in stress tolerance was investigated. We also constructed translational fusions of AlgX with LacZ and PhoA, respectively, in order to determine the subcellular localization of AlgX, which was also confirmed by immunogold-labelling and electron microscopy.

Materials and Methods

Bacterial strains, plasmids and growth of bacteria.

Bacterial strains and plasmids are listed in Table 1. Cultivations were performed at 37 °C in Luria-Bertani (LB) broth, on LB agar plates or *Pseudomonas* Isolation Agar. If required, antibiotics were added at following concentrations for *P. aeruginosa:* 300 μg/ml of gentamycin, 300 μg/ml of

carbenicillin; and for *E. coli:* 100 µg/ml of ampicillin, 12.5 µg/ml of tetracycline, 10 µg/ml of gentamycin. When required, 1 mM IPTG was added.

Table 1: Bacterial strains, plasmids and oligonucleotides

Strains, plasmids or oligonucleotides	Relevant characteristics	References
E. coli S17-1	*recA1;* harbours *tra* genes of plasmid RP4 in the chromosome	[7]
E. coli SM10	*thi*-1, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tc::Mu (Km^r)	[8]
E. coli TOP10	F-, *araD139* Δ(*ara*, *leu*)7697, Δ*lacX74*, *galU*, *galK*, *rpsL*, *deo*R, Φ80*dlacZ*Δ*M15*, *endA1*, *nupG*, *recA1*, *mcrA*, Δ(*mrr hsdRMS mcrBC*)	Invitrogen
E. coli XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_k^-, m_k^+), *supE44*, *relA1*, λ-, *lac* [F`, *proAB, lacI*^{q}, *lacZ*Δ*M15*, Tn*10*(Tc^r)]	[9]
E. coli MC4100	F^- *araD*139 Δ(*argF-lac*)U169 *rpsL*150 (Str^r) *relA1 flbB*5301 *deoC*1 *ptsF*25 *rbsR*	[10]
E. coli DH5α	F´/*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacIZYA argF*) U169 *deo*R (Φ80*dlac*Δ(*lacZ*)M15)	[11]
P. aeruginosa FRD1	CF-Isolate, Alg^+	[12]
P. aeruginosa PAO1	Prototroph, Alg^-	ATCC 15692
P. aeruginosa PDO300	*mucA22* mutant of *P. aeruginosa* PAO1, Alg^+	[13]
P. aeruginosa FRD1Δ*algX*	*P. aeruginosa* FRD1 Δ*algX* mutant, Alg^-	This study
P. aeruginosa PDO300Δ*algX*	*P. aeruginosa* PDO300 Δ*algX* mutant, Alg^-	This study
pBBR1MCS-5	Gm^r, *lac*POZ´	[14]
pEX100T	Ap^r, *oriT*, *sacB*, *lac*POZ´	[15]
pFLP2	Ap^r, *sacB*, broad host range Flp-expression vector	[16]
pGEM®-T Easy	Ap^r, *lac*POZ´	Promega
pPS856	Ap^r, source of *FRT*-Gm^r-cassette	[16]
pPHO7	*phoA* lacking signal peptide	[17]
pJE608	Ap^r, *lacI*^{q}, *ptac*, *lacZ* lacking its first 8 amino acids	[18]
pGEM-Δ*X*	pGEM®-T Easy containing the *FRT*-Gm^r-cassette flanked by a 432 bp *Eco*RV/*Bam*HI (*algX*-5´) and a 404 bp *Bam*HI/ *Eco*RV fragment (*algX*-3´) of $algX_{Pa}$	This study
pJG1-Δ*X*	pEX100T containing 1.9 kbp PCR-fragment from pGEM-ΔX	This study
pJG2-*X*	pBBR1MCS-5 containing 1.4 kbp *Hin*dIII/*Bam*HI-PCR-fragment of $algX_{Pa}$	This study
pJG4-*X*strep	pBBR1MCS-5 containing 1.5 kbp *Hin*dIII/*Bam*HI-PCR-fragment of $algX_{Pa}$ with 5´-terminal strep tag II	This study
pBBR1MCS-5:algX(Δstop)	pBBR1MCS-5 containing 1.4 kbp *Hin*dIII/*Bam*HI-PCR-fragment of $algX_{Pa}$ without stop codon	This study
pBBR1MCS-5:algX-phoA	pBBR1MCS-5:algX(Δstop) containing the 1.2 kbp *Xba*I/*Bam*HI-fragment (*phoA*) derived from pHO7	This study
pBBR1MCS-5:algX-lacZ	pBBR1MCS-5:algX(Δstop) containing 3.0 kbp *Xba*I/*Bam*HI-fragment (*lacZ*) derived from pJE608	This study
Oligonucleotides		
*algX*1N-EcV	5´-GGCATGATATCATGAAAACCCGCACTTCCCGAC-3´	This study
*algX*1C-Ba	5´-AACTGGATCCGTAGTTCTTCTTCGCCAGCTC-3´	This study
*algX*2N-Ba	5´-GGATGGATCCCAGAAGAGCTTCTACCGCCAG-3´	This study
*algX*2C-EcV	5´-ACTCGATATCTGGCAGATGCTGGCCTGGACCTC-3´	This study
*algX*N-HiSDNd	5´-CCAAAGCTT*AGGAC*CCAGACATATGAAAACCCGCAC-3´	This study
*algX*C-Ba	5´-CTTGGATCCTTACCTCCCGGCCACCGACTGGCTG-3´	This study
*algX*C-BaStrepII	5´-ATGGATCCTTATTTTTCGAACTGCGGGTGGCTCCACCT CCCGGCCACCGACTGGCTG-3´	This study
*algX*C(Δstop)	5`-AAAAGGATCCCTCCCGGCCACCGACTGGCTG-3´	This study

Isolation, analysis and manipulation of DNA.

All genetic techniques were performed as described [19]. Constructions of new plasmids were confirmed by DNA sequencing using the model 4000L automatic sequencer LI-COR (MWG-Biotech).

Generation of isogenic algX-knock out mutants and plasmid constructions.

All oligonucleotides used in this study are summarized in Table 1. PCR was performed with high-fidelity *Pfx* polymerase. The isogenic *algX*-knock out mutants were generated as previously described [20]. The inserted gentamycin cassette was removed using plasmid pFLP2 as described elsewhere [16]. The plasmid pJG2-*X* was constructed by amplifying *algX* from *P. aeruginosa* FRD1 genomic DNA using primer pair *algX*N-HiSDNd/*algX*C-Ba and subsequent subcloning into the *Hind*III/*Bam*HI sites of pBBR1MCS-5. Plasmid pJG4-*X*strep was constructed as pJG2-X but the oligonucleotide *algX*C-Ba was replaced by *algX*C-BaStrepII encoding the Strep-tag II, which consists of 8 amino acids (WSHPQFEK), that bind to Strep-Tactin. The gene *algX* was amplified by PCR using *Pfx* polymerase and primers *algX*N-HiSDNd and *algX*C(Δstop). The corresponding 1.5 kbp PCR fragment was cloned into vector pGEM-T_{Easy} (Promega), resulting in plasmid pGEM:algX(Δstop). After *Hin*dIII and *Bam*HI hydrolysis, the resulting 1.4 kbp fragment was inserted into *Hin*dIII/*Bam*HI hydrolyzed vector pBBR1MCS-5, resulting in plasmid pBBR1MCS-5:algX(Δstop) (Table 1). *Xba*I-*Bam*HI fragments of vectors pPHO7 and pJE608 were inserted into *Xba*I-*Bam*HI restricted pBBR1MCS-5:algX(Δstop) to create translational PhoA and LacZ fusions, respectively, as previously described [21].

Uronic acid assay and EPS purification.

The concentration of uronic acids was determined as previously described [20]. The concentrations of uronic acid-positive material, including alginate, were directly determined from isopropanol precipitates of supernatant fractions of planktonic *P. aeruginosa* cultures.

Preparation of crude extracts and periplasmic extracts.

For crude extract preparation, cells of an overnight culture of *P. aeruginosa* FRD1Δ*algX* harbouring pJG4-*X*strep were harvested, washed with sterile saline (0.9 % NaCl, w/v) and dissolved in 100 mM Tris-HCl buffer, pH 8.0 containing DNase I and disrupted by French press treatment. Intact cells were removed by centrifugation at 13,000 rpm for 10 min. For solubilisation of the cytoplasmic membrane 0.1 %, v/v Triton X-100 was added to the crude extract and incubated for 30 min at room temperature.

For preparation of the periplasmic extract harvested cells were dissolved in 50 mM Tris-HCl, pH 8.0, 0.2 M $MgCl_2$ (5 ml buffer g^{-1} wet weight). The suspension was incubated for 10 min at 37 °C with shaking followed by incubation on ice for 15 min. This treatment was performed twice. After centrifugation periplasmic proteins were localized in the supernatant.

MALDI-TOF mass spectrometry.

Mass spectrometric analyses were carried out on a MALDI VOYAGER DE-PRO time of flight mass spectrometer from PerSeptive BioSystems (Framingham, MA) utilising a nitrogen laser, emitting at 337 nm, and an accelerating voltage of 25 kV. Measurements were performed in the delayed extraction mode using a low mass gate of 2000. The mass spectrometer was used in the positive ion detection and linear mode. Samples of the digestion mixture were placed directly on a 100-position sample plate, and allowed to air-dry after the addition of an equal volume of saturated solution of 3,5-dimethoxy-4-hydroxycinnaminic acid (sinapinic acid) in 50% acetonitrile and 0.3% TFA.

Purification and identification of AlgX.

Gravity flow *Strep*-Tactin® Sepharose-Columns (IBA, Göttingen, Germany) with 1 ml column volume were employed for purification of AlgX-Strep-tag II-fusion protein from crude extracts or periplasmic extracts. The purification was performed according to manufacturers' instructions. Fractions were subjected to SDS-PAGE [19] and proteins were stained with Coomassie Brilliant Blue R-250. MALDI-TOF/MS analysis was performed as described above.

Electron microscopy.

Cells were prepared as described by Walther-Mauruschat et al. [22]. For the post-embedding and immunogold labeling of AlgX-strep-tagII, cells were embedded in Lowicryl K4M (Lowri) as described by Roth et al. [23], except ethanol was replaced by methanol. Strep-tag II specific monoclonal mouse antibodies served as primary antibodies and goat-anti-mouse antibodies with 5 nm gold particles were used as secondary antibodies for immunodetection. *P. aeruginosa* FRD1Δ*algX* harbouring pJG4-*X*strep was used. As negative control *P. aeruginosa* FRD1Δ*algX* was used. Micrographs were taken with a Philips EM301 electron microscope at an acceleration voltage of 80 kV.

Alkaline phosphatase and *β*-galactosidase assays.

Alkaline phosphatase assay was performed as described elsewhere [24]. The β-galactosidase assay was performed as described elsewhere [18]. One Unit corresponds to the conversion of 1 μmol substrate per min and mg of cell dry mass at 37 ºC.

Susceptibility to killing with reactive oxygen intermediates and heat.

Sensitivity to reactive oxygen was performed as described elsewhere [5]. For heat sensitivity *P. aeruginosa* FRD1 and *P. aeruginosa* FRD1Δ*algX* were grown under shaking conditions in 20 ml LB medium in 300 ml flasks at 37 °C or 42 °C, respectively. Growth was determined by measuring OD_{600} over a period of 13 h.

Results and Discussion

Generation of a marker-free isogenic *algX*-knock out mutant in *P. aeruginosa* FRD1 and phenotypic analysis.

In order to investigate the functional role of AlgX in alginate biosynthesis we generated an isogenic, marker-free *algX*-knock out mutant by deletion of 573 bp of the chromosomal *algX* in *P. aeruginosa* FRD1 using homologous recombination. In culture supernatant of *P. aeruginosa* FRD1Δ*algX* we detected 92.4 % less uronic acids (0.18 μg/ml) as compared to *P. aeruginosa* FRD1 (2.43 mg/ml). In the precipitated EPS, a decrease of 98.1 % was found, indicating a major role in formation of polymerized uronic acids. In the wildtype EPS an uronic acid concentration of 883.3 μg/ml culture supernatant was measured whereas for the mutant 16.6 μg precipitated uronic acids per ml supernatant were detected. The loss of alginate production in *algX* mutants based on insertional inactivation was already suggested elsewhere [2, 6]. The mutant generated in this study contains a deletion in *algX* but no insertion which might exert polar effects.

Complementation of the *P. aeruginosa* FRD1 *algX* knock-out mutant.

To verify that the inability of alginate biosynthesis is not a result of polar effects on genes downstream of *algX* within the alginate biosynthesis cluster we constructed plasmid pJG2-*X*, which contains only the coding region of *algX* from *P. aeruginosa* FRD1. Plasmid pJG2-*X* restored alginate production in *P. aeruginosa* FRD1Δ*algX* to wildtype level leading to the characteristic mucoid phenotype indicating no polar effects of the deletion and requirement of intact AlgX for alginate biosynthesis.

Purification of AlgX and AlgX-MucD (AlgY) protein complex.

In order to purify native AlgX the Strep-tag II was translationally fused at the C terminus. Functionality of AlgX-Strep-tag II was indicated by full restoration of alginate production in *P. aeruginosa* FRD1Δ*algX*. To analyse whether this C-terminal fusion has impact on alginate composition, isolated alginate was subjected to ^{1}H-NMR analysis. No differences could be detected (data not shown). Crude extracts and periplasmic fraction were subjected to Strep-Tactin affinity chromatography in order to purify AlgX-Strep-tag II. SDS-PAGE and MALDI-TOF/MS analysis showed that AlgX with an apparent molecular weight of 53 kDa could be purified from both fractions (Fig. 1, Table 2).

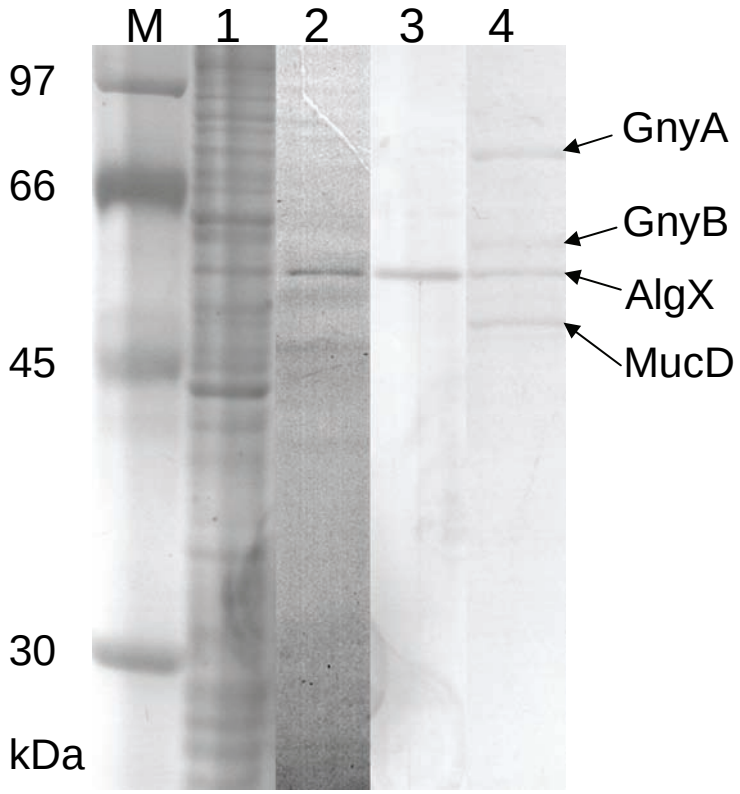

Figure 1: **SDS-PAGE analysis of purified AlgX-Strep-tag II by affinity chromatography. Lane M, molecular weight standard; lane 1, wash fraction; lane 2, AlgX from crude extract of *P. aeruginosa* FRD1Δ*algX* harbouring pJG4-*Xstrep*; lane 3, AlgX from periplasmic extract of *P. aeruginosa* FRD1Δ*algX* harbouring pJG4-*Xstrep*; lane 4, AlgX from crude extract of *P. aeruginosa* FRD1Δ*algX* harbouring pJG4-*Xstrep* treated with 0.1 % (v/v) Triton X-100.**

A co-purified protein with an apparent molecular weight of 16 kDa was identified as biotin carboxyl carrier protein (PA4847) by MALDI-TOF/MS analysis. This protein is known to be co-purified, when the Strep-Tactin matrix is used, because of its biotinylated state. Since AlgX was proposed to interact with other proteins presumably integrated in the cytoplasmic membrane

and involved in scaffold formation as well as alginate biosynthesis, we solubilized the cytoplasmic membrane with the non-ionic detergent Triton X-100. Strep-Tactin affinity chromatography showed that besides the 53 kDa and 16 kDa proteins, additional proteins with an apparent molecular weight of 71 kDa, 57 kDa and 50 kDa, respectively, were co-purified. MALDI-TOF/MS analysis led to identification of the three biotinylated proteins, PA2012 (GnyA; 71.3 kDa), PA2014 (GnyB; 57 kDa) and the BCCP (PA4847; 16.6 kDa). Since, these proteins bind to the Strep-Tactin matrix in the absence of AlgX-Strep-tag II, these proteins were directly binding to the matrix and not via interaction with AlgX (data not shown). The co-purified 50 kDa protein was identified as non-biotinylated MucD (AlgY; Table 2), which does not directly interact with the matrix but binds via specific interaction with AlgX (data not shown). A 1:1 stoichiometry based on protein band intensities was observed. Since treatment with Triton X-100 was required for co-purification of MucD (AlgY), MucD (AlgY) might either interact with the cytoplasmic membrane or the protein-protein interaction has to be stabilized by this non-ionic detergent. It is still unclear how MucD (AlgY), which is involved in alginate biosynthesis and stress tolerance, exerts its function. AlgX might control the function of MucD (AlgY) via protein-protein interaction. In the absence of AlgX the function of MucD (AlgY) might be impaired with respect to alginate biosynthesis and/or protein scaffold formation considering the putative chaperone and serine protease function of MucD (AlgY).

Table 2: Tryptic peptide fragments identified by MALDI-TOF/MS analysis leading to the identification of AlgX and MucD (AlgY) from *P. aeruginosa*

Protein	Identified peptide fragments
AlgX	T75-R91, T81-R91, T81-R94, L123-K137, A129-R146, F147-K164, Q195-K210, I319-K333, Q362-R377, N365-R377, A416-R428
MucD (AlgY)	G58-R73, G58-R78, E93-R125, V168-K193, S196-R238, K399-K434, S400-K434

Subcellular localisation of AlgX.

Analysis of AlgX using SignalP 3.0 [25] predicted a periplasmic localisation based on the presence of a putative signal peptide, whereas the program TopPred 2 [26] anticipated a membrane attached position, with the C terminus at the cytoplasmic site and the N terminus located at the periplasmic site (data not shown). A hydrophobicity analysis using ProtScale [27] showed a hydrophobic sequence at the AlgX N terminus indicating the signal peptide (Fig. 2).

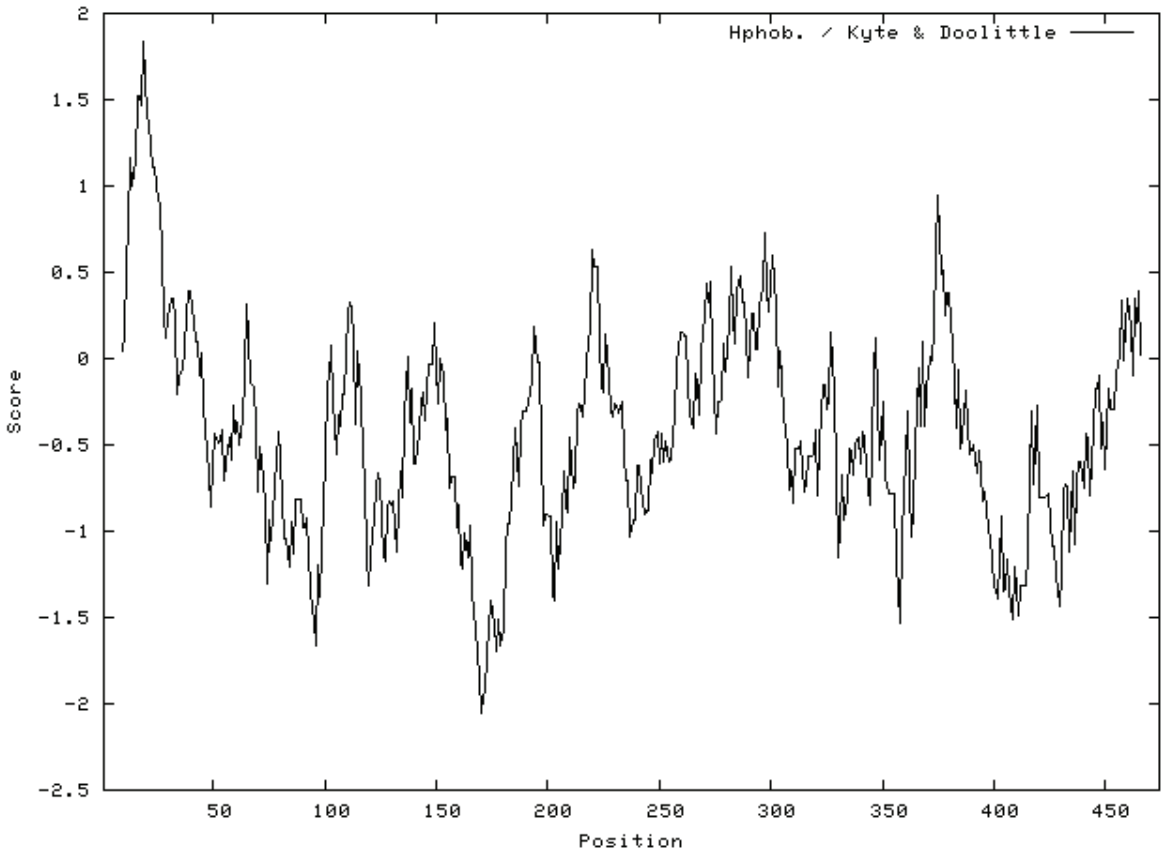

Figure 2: Hydrophobicity plot of AlgX using the program ProtScale [25] and window size 17.

Immunogold-labelling and electron microscopy led to the suggestion that AlgX-strep-tag II is located close to the cytoplasmic membrane at the cytoplasmic site (Fig. 3).

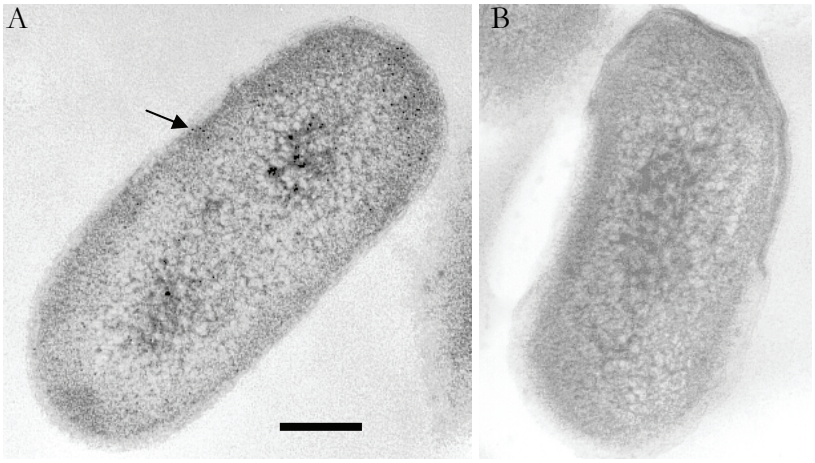

Figure 3: Immunoelectron microscopic localization of AlgX in *P. aeruginosa* FRD1Δ*algX* harbouring pJG4-*Xstrep* (A) and only *P. aeruginosa* FRD1Δ*algX* (B). Arrow indicates position of a gold particle. Bar, 0.2 µm.

Expression of plasmid pBBR1MCS-5:algX-lacZ in *P. aeruginosa* and *E. coli* MC4100 showed no β-galactosidase activity, but expression of plasmid pBBR1MCS-5:algX-phoA in *P. aeruginosa*

and *E. coli* DH5α revealed alkaline phosphatase activity. The alkaline phosphatase activity of the AlgX-PhoA fusion in *P. aeruginosa* and *E. coli* DH5α harbouring pBBR1MCS-5:algX-phoA was 21.788 ± 1.468 U/mg and 7.96 ± 0.262 U/mg, respectively. These values represent the mean values of three independent measurements including ± as standard deviation. *P. aeruginosa* and the respective *E. coli* strains harbouring pBBR1MCS-5 were used as negative control.

The plasmids encoding the respective fusion proteins restored alginate production in *P. aeruginosa* FRD1Δ*algX* suggesting that the C terminus of AlgX is located in the periplasm but AlgX might interact via its periplasmic N-terminal domain with the periplasmic MucD (AlgY). This was also supported by hydrophobicity analysis which indicated that the processed AlgX is a hydrophilic protein (Fig. 2).

Susceptibility to killing with reactive oxygen intermediates and heat.

To investigate whether AlgX exerts its function through interaction with MucD (AlgY), which homologues in *E. coli* are involved in stress tolerance, we measured the sensitivity of *P. aeruginosa* FRD1Δ*algX* to reactive oxygen intermediates and heat. *P. aeruginosa* PAO1 *mucD* (*algY*) mutant showed a higher sensitivity to H_2O_2 but not to paraquat [5]. However the absence of AlgX in *P. aeruginosa* FRD1Δ*algX* did not impact on sensitivity to H_2O_2, but caused an about 17% increase in susceptibility to paraquat, when compared with strain FRD1. In order to determine the specific role of AlgX in susceptibility towards paraquat, we generated an additional and analogous *algX*-knock out mutant of the alginate overproducing strain *P. aeruginosa* PDO300. This mutant *P. aeruginosa* PDO300Δ*algX* showed an alginate-negative phenotype as was found in *P. aeruginosa* FRD1Δ*algX* (data not shown). *P. aeruginosa* PDO300 and *P. aeruginosa* PDO300Δ*algX* showed no significant difference regarding susceptibility to paraquat (data not shown). These data suggested that AlgX is not specifically involved in oxidative stress tolerance. Phage induction was observed in *P. aeruginosa* FRD1 upon exposure to paraquat, indicated by a plaque formation surrounding the paraquat-mediated killing zone. The plaques appeared in the area beyond the killing zone at lower paraquat concentrations which enabled growth of *P. aeruginosa* cells. No phage induction occurred, i.e. no plaques were observed in *P. aeruginosa* FRD1Δ*algX*, which initially suggested a function of AlgX in the paraquat-mediated phage induction process. However, phage induction was also observed in *P. aeruginosa* PDO300Δ*algX* as well as in *P. aeruginosa* PDO300. Thus, it can be excluded that AlgX is specifically involved in paraquat-mediated phage induction. The heat challenge experiments revealed no difference in the sensitivity to heat killing. Thus in the absence of AlgX the MucD (AlgY) function related to heat stress tolerance is not impaired.

Conclusions

Complementation of the first marker-free *algX* deletion mutant by the *algX* gene indicated an essential role of AlgX in alginate formation. Analysis of reporter gene fusions strongly suggested that AlgX is localized in the periplasm and the C terminus is not required for protein function. Immunoelectron microscopy studies indicated a subcellular localization close to the membrane. The C-terminal strep-tag II fusion did not interfere with AlgX function and enabled the first purification of native AlgX. An AlgX-MucD (AlgY) protein complex could be purified, when stabilized with the Triton X-100, suggesting that AlgX functions through protein-protein interaction with MucD (AlgY), a protein involved in stress tolerance. Evidence for a specific role of AlgX in oxidative and heat stress tolerance could not be obtained.

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References

[1] **B. H. A. Rehm**, Alginates from bacteria, in: E.J. Vandamme, S. DeBaets, A. Steinbüchel (Eds.), Polysaccharides I, Wiley VCH, Weinheim, 2002, pp. 179-211 (5).

[2] **S. R. Monday, N.L. Schiller**, Alginate synthesis in *Pseudomonas aeruginosa*: the role of AlgL (alginate lyase) and AlgX, J. Bacteriol. **178** (1996), 625-632.

[3] **B.Lipinska, O. Fayet, L. Baird, C. Georgopoulos**, Identification, characterization, and mapping of the *Escherichia coli htrA* gene, whose product is essential for bacterial growth only at elevated temperatures, J. Bacteriol. **171** (1989), 1574-1584.

[4] **K. Johnson, I. Charles, G. Dougan, D. Pickard, P. O´Gaora, G. Costa, T. Ali, I. Miller, C. Hormaeche**, The role of a stress-response protein in *Salmonella typhimurium* virulence, Mol. Microbiol. **5** (1991), 401-407.

[5] **J.C. Boucher, J. Martinez-Salazar, M.J. Schurr, M.H. Mudd, H. Yu, V. Deretic**, Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA, J. Bacteriol. **178** (1996), 511-523.

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

[7] **R. Simon, U. Priefer, A. Pühler**, A broad host range mobilization system for *in vivo* genetic engineering, transposon mutagenesis in Gram-negative bacteria, Bio-Technology **1** (1983), 784-791.

[8] **V. De Lorenzo, K.N. Timmis**, Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons, Methods Enzymol. **235** (1994), 386-405.

[9] **W.O. Bullock, J.M. Fernandez, J.M. Stuart**, XL1-Blue a high efficiency plasmid transforming *recA Escherichia coli* strain with β-galactosidase selection, BioTechniques **5** (1987), 376-379.

[10] **B.L. Wanner**, Bacterial alkaline phosphatase clonal variation in some *Escherichia coli* K-12 *pho*R mutant strains. J. Bacteriol. **168** (1986), 1366-1371.

[11] **E.A. Raleigh, K. Lech, R. Brent, F.M. Ausubel, et al.** (Eds.), Current Protocols in Molecular Biology, Publishing Associates and Wiley Interscience; New York, 1989 [Unit 1.4.]

[12] **D.E. Ohman, A.M. Chakrabarty**, Genetic mapping of chromosomal determinants for the production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate, Infect. Immun. **33** (1981), 142-148.

[13] **K. Mathee, O. Ciofu, C.K. Sternberg, P. Lindum, J. Campbell, P. Jensen, A. Johnsen, M. Givskov, D.E. Ohman, S. Molin, N. Høiby, A. Kharazmi**, Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung, Microbiology **145** (1999), 1349–1357.

[14] **M.E. Kovach, P.H. Elzer, D.S. Hill, G.T. Robertson, M.A. Farris, R.M. Roop 2nd, K.M. Peterson**, Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes, Gene **166** (1995), 175-176.

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

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

[17] **C. Gutierrez, J.C. Devedjian**, A plasmid facilitating *in vitro* construction of *phoA* gene fusions in *Escherichia coli*, Nucleic Acid Res., **17** (1989), 3999-4005.

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

[19] **Sambrook, J., Fritsch, E. F. und T. Maniatis**, Molecular cloning: A Laboratory Manual, second ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory Press, NY, 1989.

[20] **T.H. Pham, J.S. Webb, B.H.A. Rehm**, The role of polyhydroxyalkanoate biosynthesis by *Pseudomonas aeruginosa* in rhamnolipid and alginate production as well as stress tolerance and biofilm formation, Microbiology **150** (2004), 3405-3413.

[21] **U. Remminghorst, B.H.A. Rehm**, *In vitro* alginate polymerisation and functional role of Alg8 in alginate production by *Pseudomonas aeruginosa*, Appl. Environ. Microbiol. (2006) in press.

[22] **A. Walther-Mauruschat, M. Aragno, F. Mayer, H. G. Schlegel**, Micromorphology of Gram-negative hydrogen bacteria. II. Cell envelope, membranes, and cytoplasmic inclusions, Arch. Microbiol. **114** (1977), 101-110.

[23] **J. Roth, M. Bendayan, E. Carlemalm, W. Villiger, M. Garavito**, Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue, J. Histochem. Cytochem**. 29** (1981), 663-671.

[24] **C. Daniels, C. Vinderampulle, R. Morona**, Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy), Mol. Microbiol. **28** (1998), 1211-1222.

[25] **J.D. Bendtsen, H. Nielsen, G. von Heijne, S. Brunak**, Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340** (2004), 783-795.

[26] **G. von Heijne**, Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule, J. Mol. Biol. **225** (1992), 487-494.

[27] **E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvand, M.R. Wilkins, R.D. Apple, A. Bairoch**, Protein identification and analysis tools on the ExPASy server, in: J.M. Walker (Ed.), The Proteomics Protocols Handbook, Humana Press, 2005, pp. 571-607.

Chapter VII

Comprehensive summary and discussion

Project aims

The aim of this thesis was to investigate the requirement of the two alginate biosynthesis proteins Alg8 and Alg44 for alginate production in *P. aeruginosa*. For this purpose, the respective genes should be knocked out without generating any downstream effect on other genes within the biosynthesis cluster. Furthermore, complementation studies should be performed with PCR fragments of the designated open reading frames to overcome the problems of earlier studies that were associated with the use of genomic subfragments and therefore the inability to assign the complementing function to only one ORF (Wang et al. 1987; Maharaj et al. 1993). Besides the requirement of the two proteins for biosynthesis, their topology and subcellular localization should be analyzed in order to create a more fitting model of the alginate polymerisation/modification and export process. This would elucidate whether or not there is a polymerisation or a membrane translocation of the cytosolic precursor without a fully or partially assembled multiprotein complex that bridges proteins of the cytoplasmic membrane with components of the outer membrane like the outer membrane channel AlgE. A detailed insight into the molecular mechanism of polymerisation and translocation would allow the generation of biosynthesis inhibitors which would be useful in medical applications for treatment of cystic fibrosis and associated chronic infections of *P. aeruginosa* in patients.

The results of this thesis clearly demonstrate the requirement of the analyzed genes and proteins for the alginate biosynthesis of *P. aeruginosa*. Furthermore, the findings led to the development of a novel alginate polymerisation and export model, in which polymerisation, regulation, modification and export are functions of a multi-enzyme complex that spans the periplasm and requires the involvement of membrane proteins of both the cytoplasmic membrane and the outer membrane (Fig. 1). This model and the results that led to its development are summarized and comprehensively discussed on the following pages.

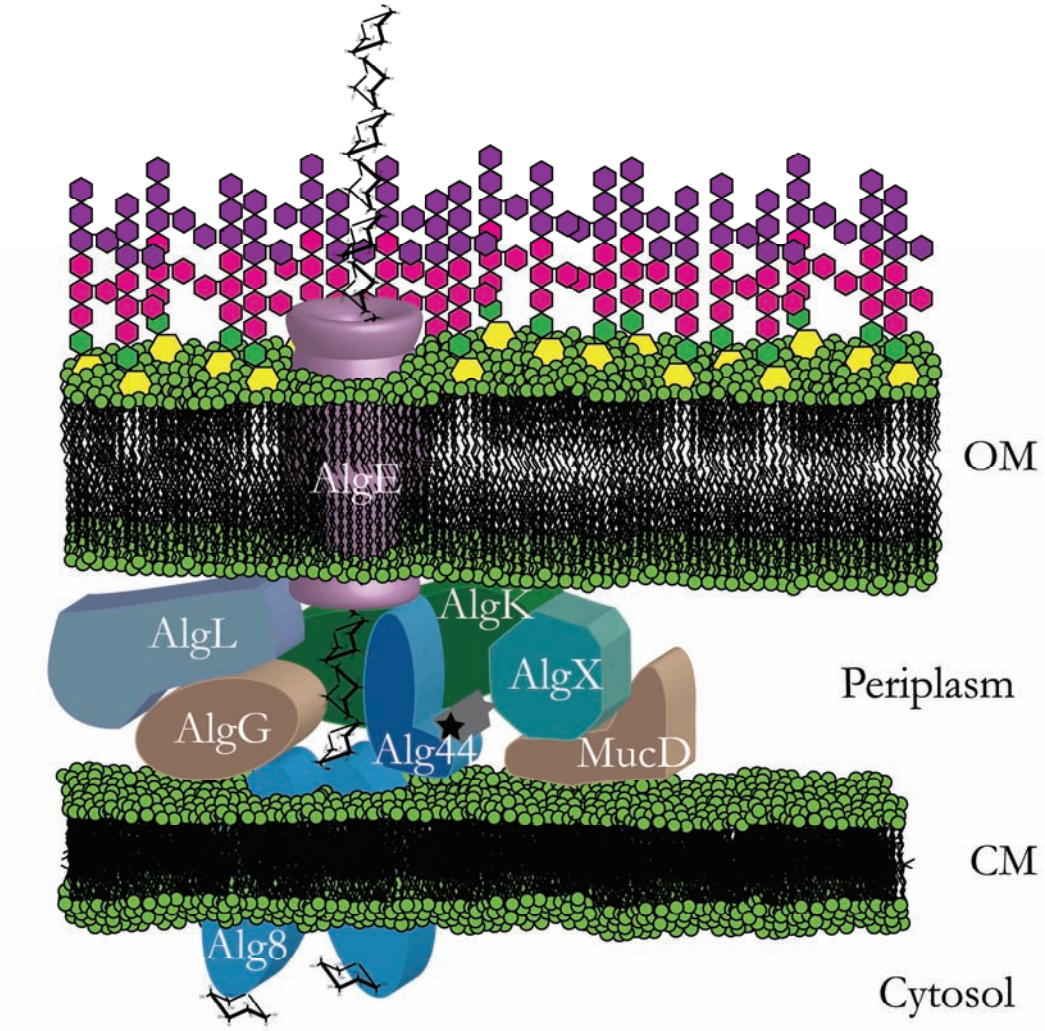

Figure 1: **Proposed model of the putative multi-enzyme complex involved in alginate polymerisation, modification and export. The star represents the effector molecule bis-(3'–5')-cyclic dimeric guanosine monophosphate (c-di-GMP). OM, outer membrane; CM, cytoplasmic membrane.**

Alg8 – Knock out and Complementation

Previous studies have shown that transposon-mediated disruptions of *alg8* could not be used to investigate the requirement for Alg8 in alginate biosynthesis. Although an alginate-negative phenotype was observed, enzymatic assays of these *alg8*-mutants showed a significant decrease in enzyme activity of proteins involved in precursor synthesis (Wang et al. 1987), suggesting that the negative phenotype might well be an effect of this decreased enzyme activity. The altered enzymatic activity of proteins encoded by genes downstream of the initial transposon insertion side suggested polar effects on the biosynthesis gene cluster caused by this insertion. In the thesis presented here, henceforth referred to as the present study, the technique of marker-free, nonpolar deletions was used to elucidate the requirement of Alg8 in alginate biosynthesis without

causing polar effects on downstream genes. The replacement of Alg8 by a functional inactive copy resulted in a mutant presenting an alginate-negative phenotype, indicating a direct involvement and requirement of Alg8 in alginate biosynthesis (Chapter II).

Studies on other alginate proteins (AlgG, AlgK, and AlgX) suggested that a nonmucoid phenotype does not necessarily correlate with the inability to polymerize alginate but with an extensive alginate degradation process caused by the alginate lyase (Gimmestad et al. 2003; Jain et al. 2003; Robles-Price et al. 2004). These researchers showed that the culture supernatants of the respective mutants contained dialyzable free uronic acids, suggesting the presence of small oligomeric or monomeric uronic acids. These oligomeric uronic acids showed a high content of unsaturated ends which are caused by the degradation of alginate by the lyase. Analysis of culture supernatants of Alg8 mutants in the present study showed no uronic acids, thus suggesting that Alg8 might be involved in an earlier step of alginate biosynthesis (Chapter II).

Earlier studies performed complementation studies by using large genomic subfragments and not by using a defined ORF. In the present study, only the ORF of the designated *alg8* gene was amplified by PCR and cloned into appropriate expression vectors (pBBR1MCS derivatives). This prevented the problems associated with the use of larger genomic subfragments, on which normally more then one ORF is located, and limited the complementing unit to the reading frame itself and no other associated sequences. Furthermore, this study used the designated *alg8* ORF of the nonmucoid strain *P. aeruginosa* PAO1, and not sequences derived from the clinical alginate-overproducing strain *P. aeruginosa* FRD1. Strain FRD1 has not been sequenced yet, so it is not fully understood which parts of the genome were modified in order to result in this stable mucoid alginate-producing strain. Although the *mucA22* allele was found to exist in strain FRD1, the phenotypical differences between this clinical isolate and the genetically engineered strain PDO300 (genetically introduced *mucA22* allele) suggested that there are larger differences between these two strains. Furthermore, the strain FRD1 is reported to be far more unstable or hyper variable with respect to its genome (Oliver et al. 2000).

The present study showed that the plasmid pBBR1MCS-5:alg8 was not stably maintained as an extrachromosomal element in strain FRD1, but was always found to be inserted into the genome. Although the exact insertion site was not investigated further, this demonstrates a further difference between the clinical isolate FRD1 and the genetically defined strain PDO300, which stably maintained all introduced plasmids. Furthermore, an increasing number of researchers found that alginate production is not stable. Even in the genetically engineered strain PDO300 many colonies lose the ability to produce alginate after several generations and revert back to the nonmucoid phenotype, although still carrying the genetically introduced mutations (Wyckoff et al. 2002; Ramos et al. 2003). This might reflect the fact that alginate production is a

high energy and carbon source consuming pathway that needs to be stopped if the organism does not environmentally benefit from the cost intensive alginate production.

The present study also showed that the constructed plasmid pBBR1MCS-5:alg8 was able to complement the *alg8* knock out mutants of *P. aeruginosa*, indicating that Alg8 is required to produce alginate. Surprisingly, the complementation observed was not only up to wildtype levels, but also exceeded the amount of alginate normally produced by the wildtype by up to 15 to 20 times. Using only the plasmid pBBR1MCS-5 without any insert showed already a twofold increase of alginate production in the wildtype strain *P. aeruginosa* PDO300. This suggested that the addition of antibiotics or - more generally spoken – the addition of environmental stress might already impact on alginate production. This finding is not surprising, because alginate production and the associated mucoid phenotype is supposedly an initial response of a nonmucoid form of *P. aeruginosa* allowing the organism to evade host immune defences during an infection (Mathee et al. 1999). Comparing the amount of alginate produced by the wildtype carrying the negative vector (pBBR1MCS-5) and the wildtype carrying the *alg8* expression vector (pBBR1MCS-5:alg8), the difference in production is around 7 times, and the differences are even bigger when comparing the *alg8* mutants. In general, the alginate production of a strain carrying additional copy numbers of *alg8* is around 10 to 15 times higher than the wildtype alginate production (Chapter II – Table 3). This might have a broader impact on alginate studies performed in the past, since for the first time it was demonstrated that the stoichiometry of alginate proteins has significant impact on the amount of alginate produced. The changes in alginate production could have easily been overlooked when complementation is only based on the restoration of the mucoid phenotype. The generation of C-terminal fusion derivatives of Alg8 (GFP, hexahistidine-tag, LacZ, or PhoA) showed no significant change in alginate production, suggesting that the C-terminus itself is not important for protein functionality.

These findings were used to functionally assign the *alg8* ORF as a complementary unit to restore alginate production, and furthermore they suggest that Alg8 might be the catalytic subunit of the alginate polymerase and even a bottleneck in alginate production. Although Alg8 might catalyze the polymerisation reaction itself, an *in vitro* polymerisation assay conducted in the present study showed that only the envelope fraction (CM, periplasm, and OM) possessed alginate polymerase activity. Indeed, only the CM membrane fraction, which would contain Alg8 based on localization studies, did not show alginate polymerisation. The polymerisation activity was significantly decreased when the non-ionic detergent Triton X-100 was omitted from assay buffers, indicating a stabilizing effect of Triton X-100 mediated envelope preparations. This finding, combined with the proposed alginate guiding, protecting and aligning scaffold in the periplasm (Bakkevig et al. 2005; Jain and Ohman 2005), proposes that the alginate polymerisation

and its export through the OM might occur as a coordinated process via the formation of a multi-protein complex involving proteins in the CM and OM, as well as the periplasm (Chapter II).

Interestingly, the overproduction of Alg8 seemed to influence the polymer properties and composition, as indicated by ^{1}H-NMR studies, although it is not understood how additional copies of Alg8 might impact on polymer composition. The degree of epimerization was significantly reduced while the degree of acetylation was slightly increased (Chapter II), which might suggest that the epimerization machinery is not able to deal with larger amounts of alginate than wildtype levels. Further experiments are needed to investigate the impact of alginate overproduction on its structure and composition.

Alg8 – Glycosyltransferases

Since alginate is primarily synthesized as polymannuronate and subsequently modified, the uronic acid-negative phenotype might be explained by the inability of polymannuronate polymerisation. The polymerisation step involves not only the polymerisation of the monomer itself but also requires the transfer of the cytosolic precursor substrate GDP-mannuronic acid either by a channel or a process that is able to transfer an activated nucleotide across a membrane component. Undecaprenol-linked intermediates have neither been found in *Pseudomonas* nor *Azotobacter* (Sutherland 1982), suggesting an undecaprenol independent process of membrane transfer. Undecaprenol carriers are involved in a variety of complex bacterial heteropolysaccharides synthesis, where repeating sugar oligosaccharides are preassembled on a carrier lipid coenzyme, undecaprenol-phosphate (Osborn and Weiner 1968; Scher et al. 1968). Another enzyme class with the capability of transferring sugar compounds are glycosyltransferases (GTs), which catalyze the transfer of sugar residues from an activated donor substrate to an acceptor molecule. In the mid 1990s, Alg8 was analyzed by hydrophobic cluster analysis, which is able to detect three-dimensional similarities in proteins showing very limited sequence relatedness (Woodcock et al. 1992), and assigned a GT because of its domain profile and its sequence similarities to other class II glycosyltransferases such as cellulose synthases (Saxena et al. 1995).

The donor molecule of those transferases is often a nucleotide diphospho sugar, but can also be a sugar phosphate or even a disaccharide. In many cases the acceptor molecule is a growing carbohydrate chain but can also be a lipid carrier or any of a variety of compounds such as steroids, flavones, or proteins that are covalently modified by glycosylation (Hundle et al. 1992). Regardless of the direction of the catalyzed reaction (i.e. synthesis or hydrolysis), GTs can

be classified according to their catalytic mechanism. The catalyzed reaction proceeds either by retention (class I) or by overall inversion (class II) of the anomeric configuration at the reaction centre (Fig. 2). A different but not very common strategy used to classify GTs uses the nucleotide recognition domain (NRD), since the type of nucleotide is the only common structure among the sugar donors and acceptors (Kapitonov and Yu 1999). The later classification method would classify Alg8 into the group NRD2 glycosyltransferases. NRD2 GTs are always membrane bound and are subdivided into a number of different subfamilies, where the cellulose synthase of *Gluconacetobacter xylinus* is the representative of the subgroup 8.

In many enzymes which appear to be directly related to Alg8 (e.g. AcsAB, cellulose synthase; NodC, chitin oligosaccharide synthase; HasA, hyaluronic acid synthase; Chs1, chitin synthase 1) the catalysis takes place by an inversion mechanism, as the donor substrates in these reactions are α-linked nucleotide diphospho sugars (e.g. GDP-mannuronic acid, UDP-glucose, UDP-N-acetylglucosamine) from which the sugar is transferred onto an acceptor molecule forming a β-linked product (alginate, cellulose, chitin).

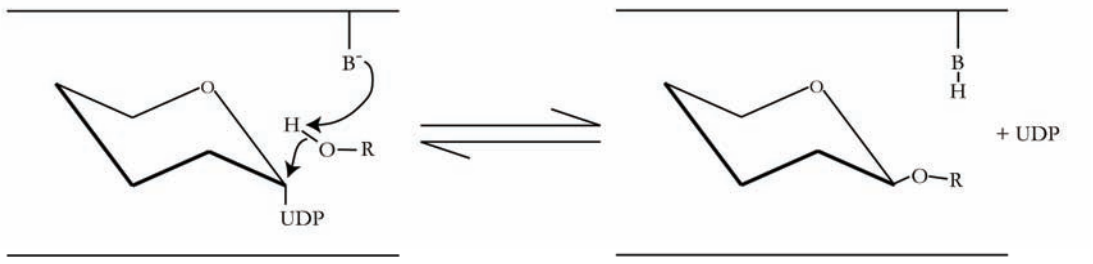

Figure 2: **Proposed glycosyltransfer of nucleotide diphospho sugars by inverting mechanism. A single nucleophilic substitution at the anomeric carbon leads to the formation of a β-linkage from a α-linked donor. ROH represents the acceptor, B the catalytic base.**

The inverting GTs most likely follow a single displacement mechanism where the acceptor performs a nucleophilic attack at carbon C-1 of the sugar donor somewhat analogous to the mechanism of inverting glycosidases. Retaining GTs, however, do not appear to operate via a two-step mechanism involving the formation of a glycosyl-enzyme intermediate analogous to glycosidases. Instead, an internal return S_Ni-like mechanism has been proposed in which the departure of the leaving group and the nucleophilic attack occur in a concerted but asynchronous manner on the same face of the glycoside (Sinnott 1990; Gibson et al. 2002).

As discussed earlier, the HCA analysis suggested similarities between Alg8 and other reported polysaccharide synthases. By analysing only the globular parts of the proteins - transmembrane segment(s) were excluded – two conserved domains could be identified. The N-terminal part (domain A) is highly conserved in all GTs and is often followed by a C-terminal

part (domain B), which is only present in processive GTs. Saxena et al. proposed a reaction model for glycosyltransferases, where the function of domain A, that is necessarily similar for both processive (like Alg8) and non-processive enzymes (like ExoM, an enzyme that adds only one sugar onto a substrate in succinoglycan synthesis), is presumably the transfer of a glycosyl residue from a nucleotide diphospho sugar to an acceptor molecular. The site 2 in domain A is always occupied by a nucleotide diphospho sugar, whereas site 3 could be occupied by any other acceptor molecule. In non-processive enzymes such as the *exo* gene products involved in succinoglycan production, the acceptor is an intermediate in the subunit assembly and the subunit chain grows from the non-reducing end.

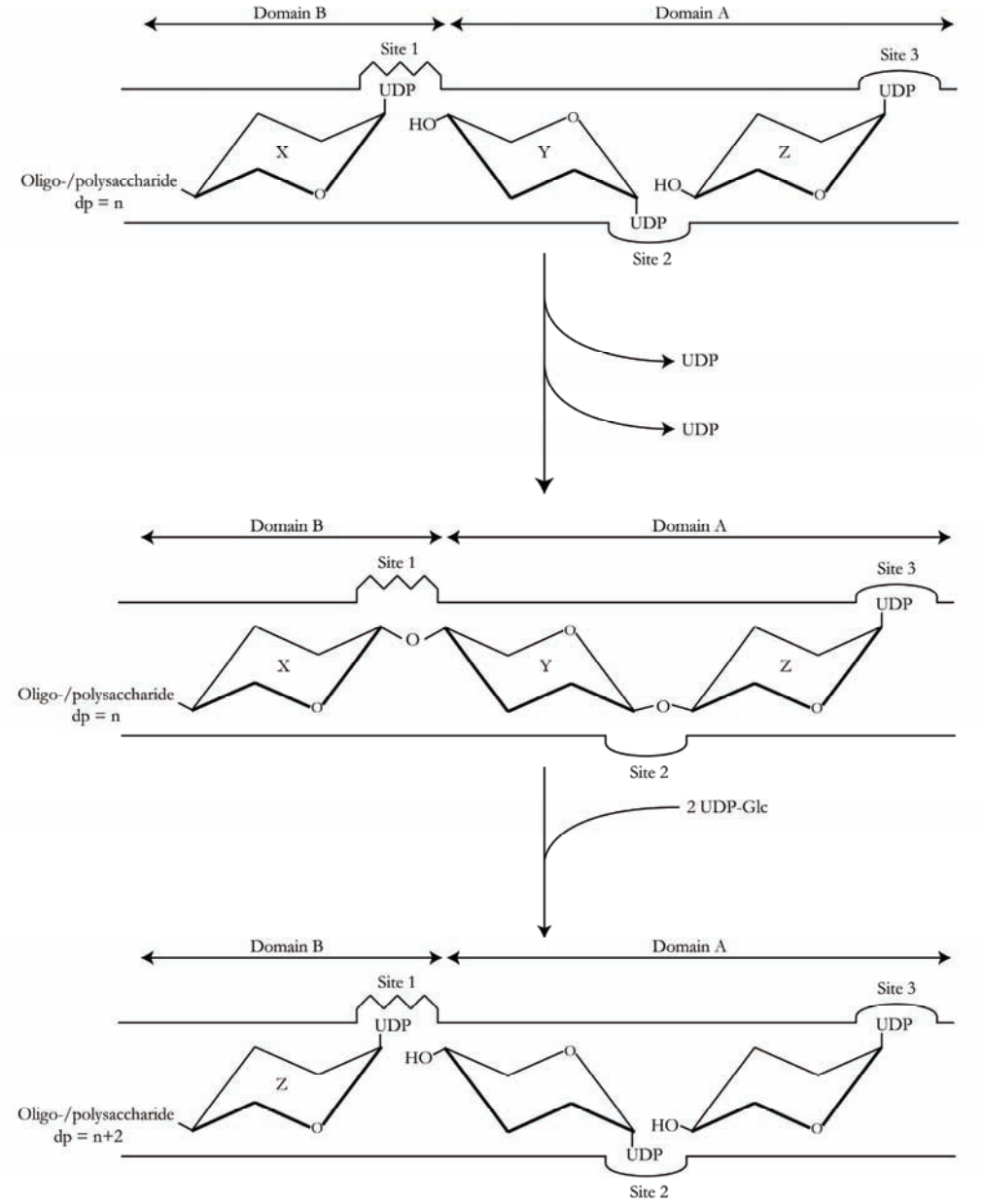

Figure 3: **Model of processive polymerisation by addition of two sugar residues resulting in growth of the polysaccharide chain from its reducing end. Modified according to Saxena (Saxena et al. 1995).**

In processive enzymes (Chitin, Cellulose synthase) that contain besides their domain A an additional domain B, the site 3 is occupied by an additional nucleotide diphospho sugar, leading to the formation of two glycosidic linkages. The oligo- or polysaccharide chain grows from its reducing end, as indicated in Fig. 3. The dual addition seems to be a plausible hypothesis for a reaction mechanism, since polymers like cellulose or alginate adopt a twofold screw axis, and simultaneous or sequential addition of two monosaccharides at the same time would allow polymer chain growth without the necessity of chain- or enzyme rotation (Saxena et al. 1995).

It is still not fully understood if there is a need for two catalytic centres in cellulose synthase and possibly other related processive synthases. The absence of duplicated active sites in a number of different processive enzymes has led some authors to suggest that there can be as much as 120º rotation about the β-1,4-linkage. Accordingly all residues could be added in a particular orientation at a single site and the alternating residues could relax into opposing orientations after they have exited the catalytic site (Delmer 1999). Two catalytic centres were thought to be required for at least those processive enzymes that catalyze the synthesis of polysaccharide chains that contain different alternating sugar residues and/or that are linked by different linkages, e.g. in the case of hyaluronan (DeAngelis 2000). Current evidence suggests that the enzymes required for their synthesis are capable of forming all of the glycosidic linkages present in these polysaccharides. Further differences in the mode of monomer addition have been described for a number of processive enzymes, where elongation of the growing chain takes place from the non-reducing end (Kamst et al. 1999; Saxena et al. 2001). This further demonstrates the diversity of mechanisms that GTs can catalyze.

Since binding of a nucleotide sugar is a common characteristic of GTs, almost all proteins with a supposed GT function contain the so called DXD motif, which is involved in nucleotide binding (Ünligil et al. 2000; Saxena et al. 2001). The motif contains two aspartic acid residues, the first (first position of the motif) is relatively variable but the second (third position of the motif) is well conserved amongst different GTs. Interestingly, the motif itself can also be reduced to only one aspartic acid residue as seen in SpsA, a GT involved in spore coat formation of *Bacillus subtilis* (Charnock and Davies 1999). Not necessarily every DXD motif found in an open reading frame is involved in nucleotide binding as demonstrated by protein LgtC which contains 4 DXD motifs, with only one shown to be involved in ribose/manganese binding (Persson et al. 2001). In many GT families the DXD sequence is preceded by at least 4 hydrophobic residues which form a short β-strand that runs through the core structure and positions the DXD motif (Ünligil et al. 2000). Although Alg8 contains two putative DXD motifs, one at Asp-182 and Asp-184, and the second at Asp-191 and Asp-193, which would fit the model of the simultaneous addition of two monomers (Fig. 3), only the second one is flanked by 4 hydrophobic residues. The 19.6%

structural similarity of Alg8 to protein SpsA (structure 1qg8a) allowed the generation of a threading model (Fig. 4) and according to this model only the second DXD motif is located within the core structure.

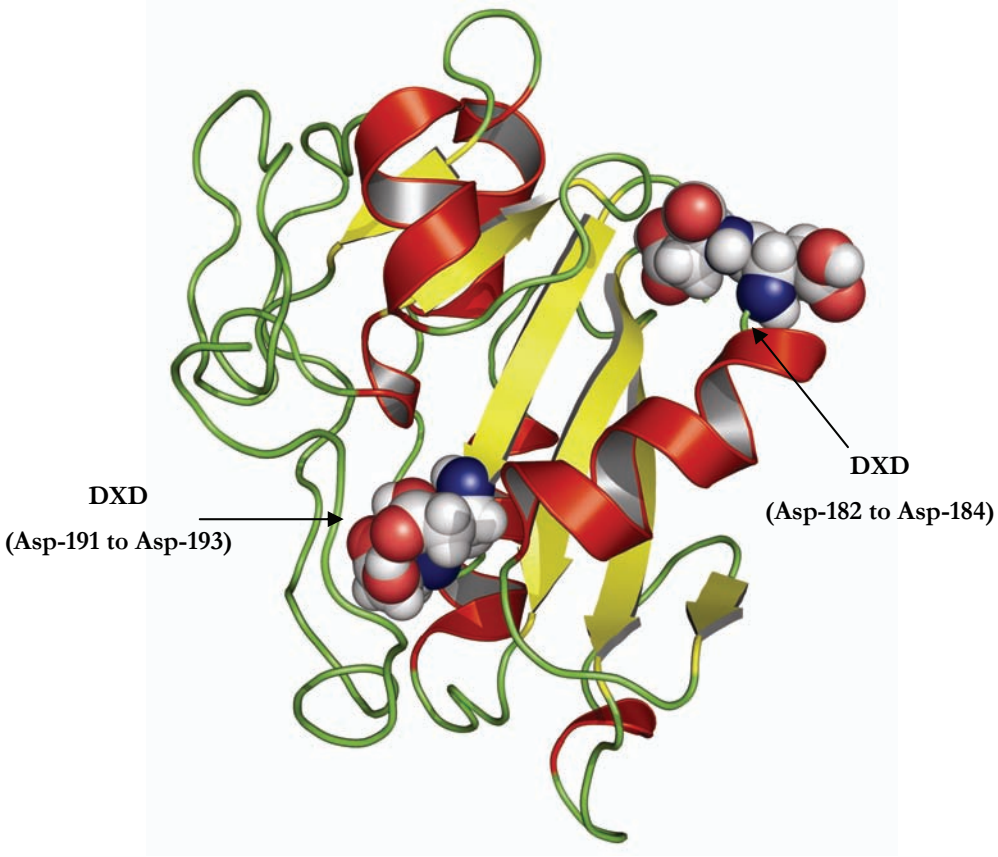

Figure 4: **Localization of the two putative DXD motifs in the Alg8 model. Only the backbone of the model is shown, and the residues are given as CPK format.**

In the present study, the site-directed replacement of Asp-182 and Asp-184 by alanine resulted in the production of reduced, but still detectable, amounts of alginate, suggesting that this DXD motif is not required for alginate production. This is in accordance with its supposed peripheral localization and its lack of a hydrophobic N-terminal flanking region (Fig. 4). The reduced amount of alginate produced by these mutants might correlate with the peripheral localization of the two residues which might impact on protein interaction or structure. Mutagenesis of the DXD motif at Asp-191 and Asp-193 showed complete abolishment of alginate production (Chapter IV), which proposes its direct involvement in catalysis. In contrast to other GTs with a more degenerated motif, each of the two aspartates seems to be important for the function. This is consistent with the finding that replacement of either of the two aspartates in the DXD motif of the yeast α-1,3-mannosyltransferase Mnn1p completely abolishes enzymatic activity (Wiggins and Munro 1998). Both of the aspartates are suggested to be involved in binding of the nucleotide sugar via interaction with a divalent cation (Mn^{2+}). The Alg8 model

(Fig. 4) illustrates the preceding β-strand, which is built by 4 hydrophobic residues, traversing the core structure. This strand positions the DXD motif at the back of a deep cleft that might act as a binding pocket. Replacement of residues in the DXD motifs have been reported to not alter the tertiary structure of the protein (Wiggins and Munro 1998), indicating that the lack of alginate production is directly caused by the loss of the functionally active carboxylic group of the respective amino acid residue.

Besides the important nucleotide sugar binding motif, all GTs contain a catalytic centre, in which a Brønsted base supposedly catalyses the connection of the two molecules involved (Fig. 2). Initial findings regarding GT SpsA suggested the involvement of a catalytic triad containing an aspartate, a histidine and a cysteine (Charnock and Davies 1999), but since none of the residues building this triad are specifically conserved amongst GTs, later studies proposed that they use either an aspartate or a glutamate as Brønsted base (Ünligil et al. 2000; Tarbouriech et al. 2001). To analyze potential candidates in Alg8, an alignment with related GTs was used to identify conserved residues which might exert Brønsted base functions. The residues Cys-247 and Asp-282 (corresponding to the triad residues in SpsA), Arg-266, Glu-285, Asp-298, Asp-299 and Lys-300 were targeted by site-directed replacement by alanine. Substitution of Cys-247 and Arg-266 did not significantly impact on alginate production, since the complemented mutants produced approximately the same amount to the wildtype Alg8 complemented mutant (Chapter IV). This showed that the residues resembling the potential catalytic triad are neither involved in enzymatic function nor in important structural motifs. The inactivation of the two potential candidates Asp-282 and Glu-285 led to a reduced alginate production, but polymer could be detected which demonstrated that the residues are not directly involved in enzyme catalysis but might impact on its structure or oligomeric state. The structural model suggested that the residues are located at the protein surface which strengthens the findings. Replacement of the residues Asp-298, Asp-299 and Lys-300 completely abolished alginate production indicating an essential role of this area for enzymatic function. Although catalytic function could not be mapped to one residue, the area might contain the Brønsted base whereas change of the direct neighborhood impacts on the tertiary structure. Although the structural model did not include these residues due to lower similarity to GT SpsA, the residues are putatively located opposite of the cleft which at least allows their involvement.

Most of the reported processive GTs (normally comprising either chitin synthases or cellulose synthases) contain an additional motif called QXXRW according to the amino acids that resemble it (Saxena and Brown 2000; Saxena et al. 2001). This motif is thought to be involved in binding of the nascent chain, but it could not be found in Alg8. This could explain the uniqueness of Alg8 with respect to monomeric substrate and growing chain and it demonstrate

that besides its similarity to cellulose and chitin synthases with respect to general GT features like nucleotide binding and general catalytic function, it also contains unique features specially required for uronic acid polymerisation or alginate production.

Alg8 – Topology and Localization

In order to analyze further the membrane topology and subcellular localization of Alg8, different reporter gene fusions were constructed in this study. The C-terminal alkaline phosphatase (PhoA) fusion derivative of Alg8 showed phosphatase activity in the insoluble membrane fraction, and selective solubilisation of the cytoplasmic membrane (CM) with *N*-laurylsarcosine showed the highest specific PhoA activity in the solubilised CM-fraction. While the C-terminal PhoA fusion showed activity, the C-terminal LacZ fusion showed no measurable β-galactosidase activity and the GFP fusion failed to produce fluorescent foci. This indicated that the C terminus of Alg8 is exposed to the periplasm, although its hydrophobicity and membrane profile showed a highly hydrophobic C-terminal part. So it appears that the untagged C-terminal end just crosses the cytoplasmic membrane and/or stays embedded in the membrane. The experimentally confirmed CM-localization further strengthened the earlier discussed HCA results, supporting the hypothesis that Alg8 is a glycosyltransferase located in the CM. The predicted topology of Alg8 initially showed a signal peptide at the N terminus and four TM domains, three of them located at the C-terminal end of the protein and one directly following the putative signal peptide cleavage site (Fig. 5).

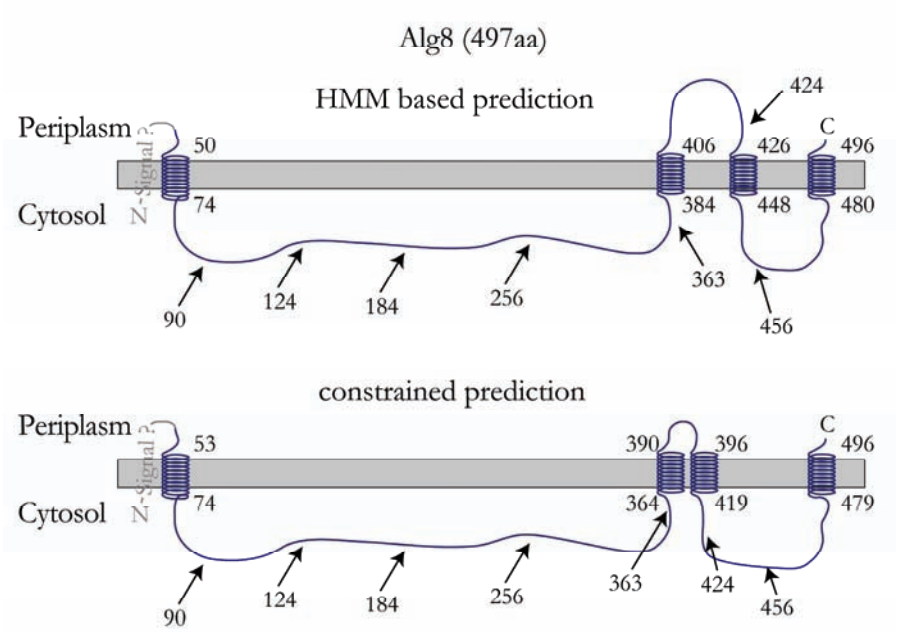

Figure 5: **Predicted membrane topology of Alg8 based on different HMM-based algorithms (Phobius (Käll et al. 2004), SMART (Schultz et al. 1998), and TMHMM2 (Moller et al. 2001)). The constrained prediction is based on Phobius using all experimentally confirmed positions. The arrows represent reporter gene fusion sites.**

The consensus model of the topological predictions indicated a large cytosolic loop and a C-terminal membrane anchoring part. The large cytosolic loop showed significant homologies to class II GTs and its cytosolic orientation fits its functions since the alginate precursor GDP-mannuronic acid is synthesized in the cytosol (Fig. 5).

Seven reporter protein fusions were generated in order to analyze the topology in detail. In order to prevent disruption of helical structures which might function as TM segments the secondary structure was reanalyzed by program jPred (Cuff and Barton 2000) which uses 6 different algorithms to predict the secondary structure. The different algorithms showed extended helical stretches in the C-terminal region of Alg8 but varied with respect to the localization of the third TM domain. The HMM based prediction showed a periplasmic loop of about 20 amino acid residues between TM 2 and TM3, whereas some of the jPred predictions indicated the possibility of 2 TM domains interspaced by only 6 periplasmic residues. This would place the fusion site 424 (FusN-424) into the cytosol, while the HMM based prediction suggested a periplasmic localization. High β-galactosidase activity of the respective LacZ fusion proteins could be detected at all 7 fusion points (Chapter IV), suggesting a cytosolic localization for all analysed sites. Elevated levels of alkaline phosphatase activity could not be detected, which confirmed the LacZ results. The first 5 fusion sites were located inside the potentially GT-comprising area, and results indicated that the entire loop with its predicted helical structures is located in the cytosol, which is a direct requirement for its supposed function in binding of the cytosolic precursor GDP-mannuronic. Using all experimentally confirmed localizations, program Phobius was used to predict the membrane topology and the constrained prediction showed that Alg8 contains at least 4 TM segments (Fig. 5). None of the Alg8 fusion proteins, with the exception of the full-length fusions, was able to complement the *alg8* mutant of *P. aeruginosa* PDO300, demonstrating the requirement of the C-terminal membrane anchoring region for catalysis. Although some tools predict a signal sequence with a likely cleavage site between residue 31 and 32, it can not be excluded that Alg8 does not contain a signal sequence hence not being N-terminally processed. But given the hydrophobic nature of the first 31 amino acid residues, in which prediction programs homogeneously indicate a TM segment, the topology of the protein would not be altered with an additional TM domain at the N terminus. Only N-terminal sequencing of the native Alg8 protein will ultimately unravel the structure of the N terminus, but that requires the purification and visualization of Alg8 which has so far not been demonstrated.

Alg8 - Impacts on biofilms

With the finding that additional copy numbers of Alg8 resulted in an increased alginate production in *P. aeruginosa* strains, the question arose as to what extent the overproduction of alginate impacts on biofilms. Although alginate is not required for the early stages of biofilm formation or the initial attachment, it is however the major matrix material contributing to matured biofilms containing different architectures (Nivens et al. 2001). The results indicated that the alginate producing strains of *P. aeruginosa* show weaker attachment to solid surfaces after 2 to 6 hours than the non-alginate producing strains (Chapter V). Polyhydroxyalkanoate (PHA) synthesis was shown to reduce biofilm formation, perhaps because it competes with the alginate or exopolymer biosynthesis for common metabolic substrates, while increasing stress tolerance (Pham et al. 2004). Analysis revealed an approximate 3-fold decrease in PHA content in the alginate-overproducing strain *P. aeruginosa* PDO300Δalg8 (pBBR1MCS-5:alg8) when compared to the alginate negative strain PDO300Δalg8 (pBBR1MCS-5). These data confirmed the previous finding that extensive alginate production reduces other biosynthesis pathways, likely due to channelling of common metabolites towards alginate biosynthesis. Furthermore, the alginate overproduction led to the extensive formation of large streamers.

Alg8 - Outlook

Many characteristics of Alg8 are still unknown. The existence of a signal sequence can only be confirmed if the protein can be visualized and analyzed when produced in its native host *P. aeruginosa*. Although this thesis endeavours to address issues regarding Alg8's membrane topology, function and catalytic activity, deeper insight into the structure can only be gained via the production and purification of the protein. It will be interesting to see how many alginate polymerizing and exporting multi-enzyme complexes are located in each cell. Are these complexes located on only one side in close proximity as seen in cellulose synthesis? Crystallographic studies could be used to gain insights into nucleotide binding and catalytic activity. Ultimately, knowledge of the alginate polymerisation process could be used to design drugs in order to prevent extensive alginate production during clinical infections of *P. aeruginosa*. On the other hand, understanding how *P. aeruginosa* synthesises alginate may allow the generation of specially engineered strains that produce a tailor-made alginate according to different commercial demands. Since Alg8 seems to be the catalytic subunit of the alginate polymerase and the bottleneck of alginate production, understanding its structure and function would have great

influence on the future of not only alginate research, but also the commercial use of alginate from its different producers with respect to different applications.

Alg44 – Knock out and Complementation

As for Alg8, the experiments dealing with Alg44 that were documented in the literature were all based on transposon studies, where random transposon mutagenesis was used to disrupt genes of interest. In order to get valid experimental results that would allow the investigation of the requirement of Alg44 for alginate biosynthesis in *P. aeruginosa*, a marker-free nonpolar deletion mutant of *alg44* was generated in this study. The resulting *alg44* deletion mutants showed a loss of alginate production as indicated by the nonmucoid appearance. In order to investigate whether the loss of alginate production is concomitant with the secretion of free uronic acids as discussed for deletions of AlgK, AlgG, and AlgX (Gimmestad et al. 2003; Jain et al. 2003; Robles-Price et al. 2004), the culture supernatants of the respective mutants were analyzed (Chapter III). Uronic acid monomers or oligomers could not be detected. Furthermore the cellular structure was analyzed by electron microscopy. Recent publications suggested that the deletion of the alginate lyase AlgL resulted in a lethal phenotype, since misfolded alginate accumulated in the periplasmic space without being controlled or degraded by the lyase (Jain and Ohman 2005). This alginate accumulation ultimately led to loss of the periplasmic space and cellular burst, suggesting that AlgL has a function in clearing the periplasmic space of misguided or misfolded alginate. In the present study, no electron microscopically detectable phenotypical differences between the wildtype and the deletion mutant could be detected. This suggests that Alg44 is involved in the polymerisation process, although the involvement might not be as a catalytic unit but more as a structural or regulatory compound.

Complementation studies conducted in this thesis used the PCR amplified designated *alg44* ORF of the nonmucoid strain *P. aeruginosa* PAO1, and the mucoid phenotype was restored after introduction of the Alg44 encoding vector into the *alg44* deletion mutant PDO300Δalg44. These findings demonstrate the requirement of Alg44 for alginate production and allowed the functional assignment of the *alg44* ORF as a complementary unit to restore alginate production (Chapter III). The level of alginate production was restored up to wildtype levels, showing that the introduction of multiple copy numbers of Alg44 does not impact on alginate production. This is in direct contrast to Alg8, where additional copy numbers of the protein significantly increased the amount of alginate produced by the organism. Different C-terminal reporter gene fusions (LacZ, PhoA, hexahistidine tag) did not significantly impact on alginate production as

each fusion protein restored alginate production approximately to wildtype levels, which led to the suggestion that the C terminus is not directly involved in protein function (Chapter III).

Alg44 – Topology and Localization

According to many previous publications, Alg44 was generally reported to be a transmembrane protein, and suggested to be – in combination with Alg8 – a putative subunit of the alginate polymerase complex (Rehm and Valla 1997; Rehm 2002). This characterization is mainly based on its hydrophobicity profile, as many HMM-based topology tools (TMHMM2, SMART, Phobius) predict a transmembrane domain at around the middle of the protein. The lack of a predicted signal sequence of Alg44 would place its N terminus into the cytosol and, considering the transmembrane domain, results in a periplasmic exposed C terminus. The reporter gene fusions supported this topology model, shown by an active PhoA fusion and an inactive β-galactosidase fusion protein. When the experimentally confirmed periplasmic C terminus was used for constrained topology predictions with the program Phobius, the predicted model was further strengthened. Immunological detection of the hexahistidine-tagged protein in the native *Pseudomonas* host as well as a recombinant *E. coli* production host revealed a protein of 42.6 kDa, which correlates with the predicted theoretical mass of 42.61 kDa of $Alg44_{6xHis}$ (Chapter III). Since the $Alg44_{6xHis}$ is able to restore the alginate producing phenotype, the protein is supposedly not N-terminally processed which further supported the topological model. Interestingly, the highest specific PhoA activity was not associated with the insoluble membrane fraction, but with the soluble membrane-free fraction. This finding did not support the predicted topology model or the presence of the predicted transmembrane domain, since it suggested a periplasmic localization of Alg44. The protein export into the periplasm is usually dependent on an N-terminal signal sequence, although there are other mechanisms of protein localization (for review see (Driessen et al. 2001)). The dehalogenases LinA and LinB from pseudomonads/sphingomonads have been reported to be localized in the periplasm without being N-terminally processed (Nagata et al. 1999).

The use of tools that perform homology searches based on predicted secondary structures can detect structural similarities to other proteins even if the primary structure shows no significant homology. The meta-prediction tool 3D-Jury (Ginalski et al. 2003) was used to analyze protein Alg44 and revealed a 16.8 % similarity of the C terminus to the structure of MexA (Chapter III). MexA is a multimeric protein which is involved in the multidrug efflux system of *P. aeruginosa*, specifically in bridging the cytoplasmic membrane transporter MexB with an outer membrane export protein OprM (Akama et al. 2004). It is considered to be a membrane

fusion protein (MFP) since it connects components in the CM with OM components. These similarities, together with the experimentally confirmed periplasmic localization of Alg44, might suggest that Alg44 has a similar function in bridging a cytoplasmic membrane protein (Alg8) with an outer membrane channel (AlgE). Hence, Alg44 might have a structural role in coordinating the alignment of the multi-protein complex that is involved in alginate polymerisation and export, while scaffold proteins AlgKGXL might be required for protection of the nascent alginate chain. Recently, a PilZ domain has been identified in the N-terminal part of Alg44 based on bioinformatical data (Amikam and Galperin 2006). The PilZ domain was thought to be involved in the binding of the important regulatory molecule, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), and recent studies experimentally confirmed the binding of the nucleotide (Bateman et al. 2004; Ryjenkov et al. 2006). C-di-GMP is a novel second messenger in bacteria that was first described as an allosteric activator of the cellulose synthase in *Gluconacetobacter xylinus* (Ross et al. 1987). It is now established that this nucleotide is almost ubiquitous in bacteria where it regulates a variety of functions including developmental transitions, aggregative behaviour, adhesion, biofilm formation, and the virulence of animal and plant pathogens (for reviews see Jenal 2004; Jenal and Malone 2006).

The level of c-di-GMP in bacterial cells is influenced or controlled by both synthesis and degradation, whereby proteins that possess a GGDEF domain are generally supposed to synthesize c-di-GMP and proteins showing an EAL or HD-GYP domain normally degrade the messenger. In the majority of these proteins either of these domains / or both are coupled with signal input domains. These signalling systems are supposed to use c-di-GMP as a second messenger to link the sensing of specific environmental stimuli to appropriate alterations in bacterial physiology and/or gene expression (Ryan et al. 2006). The availability of a protein able to recognize this important second messenger in alginate biosynthesis might indicate that alginate polymerisation is influenced by different levels of c-di-GMP, which would be analogous to cellulose biosynthesis. In the cellulose biosynthesis, c-di-GMP is suggested to bind to BcsA, the α-subunit of the cellulose synthase, whereby PilZ is located at the C terminus of the protein (Amikam and Galperin 2006). Thus, Alg44 might exert regulatory function in controlling the alginate polymerase, which would explain why no free uronic acids could be detected even though the protein is periplasmic. The lack of the regulatory compound or the signal transduction between Alg8 and Alg44 might result in the prevention of alginate biosynthesis.

Alg44 – Outlook

The interesting finding of a PilZ domain in an alginate protein opens up a new and exciting field for future research of alginate biosynthesis, as the biosynthesis itself seems to be posttranslationally controlled by the novel second messenger c-di-GMP. As a very interesting side note it needs to be stressed that Alg44 appears to be a periplasmic protein. So far, c-di-GMP is only considered to be an intracellular second messenger, but increasingly more authors are discussing the possibility of different localized pools of c-di-GMP (Ryan et al. 2006). There are speculations that c-di-GMP might also exert signalling function outside the bacterial cell, since the possibility of discrete localized pools would not necessarily mean a mass export of the nucleotide. These possibilities open up a very interesting area of research and need to be further investigated in the future. Furthermore, biochemical characterization could be used to identify important functional regions in protein Alg44, and its overproduction in the native or a heterologous host might be used to gain structural information.

AlgX– Knock out and Complementation

In order to investigate the requirement of AlgX in alginate biosynthesis, isogenic marker-free knock out mutants of strains *P. aeruginosa* PDO300 and FRD1 were generated. The mutants showed an alginate-negative, nonmucoid phenotype, and consequently uronic acid assays showed a huge decrease (92 %) of uronic acids in the culture supernatants when compared to the wildtype strain *P. aeruginosa* FRD1. This confirmed previously reported data, where an inactivation of *algX* was achieved by replacement of the 3'-end by a gentamycin cassette (Robles-Price et al. 2004). Since this study used an inactivation method that added genetic material (gentamycin cassette) into the genome in order to disrupt the ORF, we have chosen to inactivate the gene *algX* by deletion of about 550 bp in the middle of the designated ORF (Chapter VI). The marker used for screening of homologous recombination events has been excised in later experiments, finally resulting in the marker-free knock-out mutant. In contrast to deletion mutants of genes *alg8* and *alg44*, uronic acid could still be found in the culture supernatants, which confirmed earlier findings that the deletion of AlgX resulted in the secretion of small uronic acid oligomers (Robles-Price et al. 2004). Since uronic acid oligomers were found in the supernatants, the loss of the mucoid phenotype could not be explained with the loss of alginate polymerisation or transfer of the precursor. It was shown that the dialyzable uronic acid components had a high degree of unsaturated bonds which is due to degradation caused by the

alginate lyase. This suggested that AlgX is involved in the periplasmic scaffold that supposedly protects and aligns the nascent alginate chain. Deletion of proteins AlgK, AlgG and AlgX led to the secretion of free uronic acids, hence these proteins are discussed to form a protective barrier that protects the alginate chain against degradation by AlgL (Jain and Ohman 1998; Jain et al. 2003; Robles-Price et al. 2004; Jain and Ohman 2005). In the present study, complementation experiments using only the PCR amplified designated ORF of *algX* were able to restore the mucoid phenotype, indicating that the deletion had no downstream effects on the biosynthesis cluster (Chapter VI).

To further characterize AlgX, a C-terminal Strep-tag II fusion was generated. This fusion was able to restore the mucoid phenotype which indicates that the C terminus is not directly involved in protein function. NMR studies of isolated polymers showed that the introduction of the tag had no effects on alginate composition. The use of the strep-tag allowed purification of the native protein and since AlgX could be isolated from periplasmic extracts, its localization in the periplasm was suggested. The periplasmic localization of at least the C-terminus could be experimentally confirmed since C-terminal fusions to PhoA resulted in an active alkaline phosphatase protein whereas β-galactosidase activity of the LacZ fusion protein could not be detected. HMM based topological predictions and hydrophobicity analysis did not show stretches of hydrophobic elements, which would indicate the existence of transmembrane domains, so AlgX is supposedly entirely located in the periplasm. Interestingly, by using a Triton X-100 mediated solubilisation, not only AlgX but also MucD could be purified, which suggests that the non-ionic detergent might stabilize the protein-protein interaction. MucD is encoded in the genotypic switch region as part of the *mucABCD* gene cluster, and supposedly influences stress tolerance and alginate biosynthesis (Wood and Ohman 2006). MucD is a close homologue of the *E. coli* periplasmic serine protease HtrA (DegP), which is required in *E. coli* for resistance to high temperature and oxidative stress (Boucher et al. 1997; Pallen and Wren 1997). Recent findings have suggested that although *mucD* is located in the *muc* gene cluster it is not only controlled by the *algU* promoter but also has an additional promoter region. This promoter is possibly located just upstream of *mucD* in the 3'-end of *mucC* (Wood et al. 2006). Site-specific mutagenesis of the active site serine 217 of the protease led already to a loss of alginate regulation and therefore alginate production, since a functionally active MucD negatively influences alginate biosynthesis and production.

It is still unclear how MucD influences alginate biosynthesis, but interaction with AlgX suggests that protein interaction might control the function of MucD. In the absence of AlgX the putative chaperone or protease function of MucD for alginate biosynthesis and/or protein scaffold formation might be impaired. To investigate the direct involvement of AlgX in stress

tolerance or to determine if AlgX exerts its function via interaction with MucD, which then impacts on stress tolerance, the sensitivity of *algX* knock out mutants with respect to reactive oxygen intermediates and heat was analyzed. The results clearly indicate that AlgX negative mutants did not differ from the wildtype strain with respect to reactive oxygen, phage induction, or heat tolerance.

AlgX - Outlook

The absence of AlgX showed no effect on stress tolerance of *P. aeruginosa* which suggests that AlgX is not impacting on the MucD-mediated function in heat or stress tolerance. Wood and Ohman showed that loss of MucD, however, results in loss of alginate biosynthesis regulation (which leads to alginate biosynthesis) and higher sensitivity to heat (Wood and Ohman 2006). Interestingly, the serine protease function itself is important for biosynthesis regulation, which suggests an involvement of proteolytic function in the regulation process. It needs to be stated, however, that expression of the protease function impaired *mucD* (shown to deregulate and therefore activate alginate biosynthesis) from a high-copy vector led to nonmucoid wildtype phenotype (Wood and Ohman 2006). To identify the role of AlgX in alginate biosynthesis, the protein itself needs to be screened for important regions and needs to be characterized at molecular level. Overproduction of AlgX might be used to gain further insights into the structure of AlgX, which so far shows no similarities to any other known protein structure.

References

Akama, H., T. Matsuura, S. Kashiwagi, H. Yoneyama, S. Narita, T. Tsukihara, A. Nakagawa and T. Nakae (2004). Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in *Pseudomonas aeruginosa*. J. Biol. Chem. **279**(25): 25939-25942.

Amikam, D. and M. Y. Galperin (2006). PilZ domain is part of the bacterial c-di-GMP binding protein. Bioinformatics **22**(1): 3-6.

Bakkevig, K., H. Sletta, M. Gimmestad, R. Aune, H. Ertesvåg, K. Degnes, B. E. Christensen, T. E. Ellingsen and S. Valla (2005). Role of the *Pseudomonas fluorescens* alginate lyase (AlgL) in clearing the periplasm of alginates not exported to the extracellular environment. J. Bacteriol. **187**(24): 8375-8384.

Bateman, A., L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E. L. Sonnhammer, D. J. Studholme, C. Yeats and S. R. Eddy (2004). The Pfam protein families database. Nucleic Acids Res **32** (Database issue): D138-141.

Boucher, J. C., H. Yu, M. H. Mudd and V. Deretic (1997). Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect. Immun. **65**(9): 3838-3846.

Charnock, S. J. and G. J. Davies (1999). Structure of the nucleotide-diphospho-sugar transferase, SpsA from *Bacillus subtilis*, in native and nucleotide-complexed forms. Biochemistry **38**(20): 6380-6385.

Cuff, J. A. and G. J. Barton (2000). Application of multiple sequence alignment profiles to improve protein secondary structure prediction. Proteins **40**(3): 502-511.

DeAngelis, P. L. (2000). Molecular directionality of polysaccharide polymerization by the Pasteurella multocida hyaluronan synthase. J. Biol. Chem. **274**: 26557-26562.

Delmer, D. P. (1999). Cellulose Biosynthesis: Exciting times for a difficult field of study. Annu. Rev. Plant. Physiol. Plant. Mol. Biol. **50**: 245-276.

Driessen, A. J., E. H. Manting and C. van der Does (2001). The structural basis of protein targeting and translocation in bacteria. Nat. Struct. Biol. **8**(6): 492-498.

Gibson, R. P., J. P. Turkenburg, S. J. Charnock, R. Lloyd and G. J. Davies (2002). Insights into trehalose synthesis provided by the structure of the retaining glucosyltransferase OtsA. Chem. Biol. **9**(12): 1337-1346.

Gimmestad, M., H. Sletta, H. Ertesvåg, K. Bakkevig, S. Jain, S.-J. Suh, G. Skjåk-Bræk, T. E. Ellingsen, D. E. Ohman and S. Valla (2003). The *Pseudomonas fluorescens* AlgG protein, but not its mannuronan C-5-epimerase activity, is needed for alginate polymer formation. J. Bacteriol. **185**: 3515-3523.

Ginalski, K., A. Elofsson, D. Fischer and L. Rychlewski (2003). 3D-Jury: a simple approach to improve protein structure predictions. Bioinformatics **19**(8): 1015-1018.

Hundle, B. S., D. A. O'Brien, M. Alberti, P. Beyer and J. E. Hearst (1992). Functional expression of zeaxanthin glucosyltransferase from *Erwinia herbicola* and a proposed uridine diphosphate binding site. Proc. Natl. Acad. Sci. USA **89**(19): 9321-9325.

Jain, S., M. J. Franklin, H. Ertesvåg, S. Valla and D. E. Ohman (2003). The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. Mol. Microbiol. **47**(4): 1123-1133.

Jain, S. and D. E. Ohman (1998). Deletion of *algK* in mucoid *Pseudomonas aeruginosa* blocks alginate polymer formation and results in uronic acid secretion. J. Bacteriol. **180**(3): 634-641.

Jain, S. and D. E. Ohman (2005). Role of an alginate lyase for alginate transport in mucoid *Pseudomonas aeruginosa*. Infect. Immun. **73**(10): 6429-6436.

Jenal, U. (2004). Cyclic di-guanosine-monophosphate comes of age: a novel secondary messenger involved in modulating cell surface structures in bacteria? Current Opinion in Microbiology **7**(2): 185-191.

Jenal, U. and J. Malone (2006). Mechanisms of Cyclic-di-GMP Signaling in Bacteria. Annu. Rev. Genet. **40**: 385-407.

Käll, L., A. Krogh and E. L. Sonnhammer (2004). A combined transmembrane topology and signal peptide prediction method. J. Mol. Biol. **338**(5): 1027-1036.

Kamst, E., J. Bakkers, N. E. Quaedvlieg, J. Pilling, J. W. Kijne, B. J. Lugtenberg and H. P. Spaink (1999). Chitin oligosaccharide synthesis by rhizobia and zebrafish embryos starts by glycosyl transfer to O4 of the reducing-terminal residue. Biochemistry **38**(13): 4045-4052.

Kapitonov, D. and R. K. Yu (1999). Conserved domains of glycosyltransferases. Glycobiology **9**(10): 961-978.

Maharaj, R., T. B. May, S. K. Wang and A. M. Chakrabarty (1993). Sequence of the *alg8* and *alg44* genes involved in the synthesis of alginate by *Pseudomonas aeruginosa*. Gene **136**(1-2): 267-269.

Mathee, K., C. Sternberg, O. Ciofu, P. Jensen, J. Campbell, M. Givskov, D. E. Ohman, N. Hoiby, S. Molin and A. Kharazmi (1999). Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. Microbiol. **145**: 1349-1357.

Moller, S., M. D. Croning and R. Apweiler (2001). Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics **17**(7): 646-653.

Nagata, Y., A. Futamura, K. Miyauchi and M. Takagi (1999). Two different types of dehalogenases, LinA and LinB, involved in gamma-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26 are localized in the periplasmic space without molecular processing. J. Bacteriol. **181**(17): 5409-5413.

Nivens, D. E., D. E. Ohman, J. Williams and M. J. Franklin (2001). Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. J. Bacteriol. **183**(3): 1047-1057.

Oliver, A., R. Canton, P. Campo, F. Baquero and J. Blazquez (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. Science **288**(5469): 1251-1254.

Osborn, M. J. and I. M. Weiner (1968). Biosynthesis of a bacterial lipopolysaccharide. VI. Mechanism of incorporation of abequose into the O-antigen of *Salmonella typhimurium*. J. Biol. Chem. **243**(10): 2631-2639.

Pallen, M. J. and B. W. Wren (1997). The HtrA family of serine proteases. Mol. Microbiol. **26**(2): 209-221.

Persson, K., H. D. Ly, M. Dieckelmann, W. W. Wakarchuk, S. G. Withers and N. C. Strynadka (2001). Crystal structure of the retaining galactosyltransferase LgtC from *Neisseria meningitidis* in complex with donor and acceptor sugar analogs. Nat. Struct. Biol. **8**(2): 166-175.

Pham, T. H., J. S. Webb and B. H. A. Rehm (2004). The role of polyhydroxyalkanoate biosynthesis by *Pseudomonas aeruginosa* in rhamnolipid and alginate production as well as stress tolerance and biofilm formation. Microbiology **150**(Pt 10): 3405-3413.

Ramos, D., A. Heydorn, C.-L. Koh and K. Mathee (2003). Loss of alginate production in mucoid *Pseudomonas aeruginosa* occurs via deregulation of the alternative sigma factor AlgT. Proceedings of the National Conference on Undergraduate Research (NCUR), Salt Lake City, Utah.

Rehm, B. H. A. (2002). Alginates from Bacteria. Biopolymers. E. J. Vandamme, S. DeBaets and A. Steinbüchel, Wiley VCH. **Polysaccharides:** 179-211.

Rehm, B. H. A. and S. Valla (1997). Bacterial alginates: biosynthesis and applications. Appl. Microbiol. Biotechnol. **48**(3): 281-288.

Robles-Price, A., T. Y. Wong, H. Sletta, S. Valla and N. L. Schiller (2004). AlgX is a periplasmic protein required for alginate biosynthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **186**(21): 7369-7377.

Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinberger-Ohana, R. Mayer, S. Braun, E. de Vroom, G. A. van der Marel, J. H. van Boom and M. Benziman (1987). Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid. Nature **325**(6101): 279-281.

Ryan, R. P., Y. Fouhy, J. F. Lucey and J. M. Dow (2006). Cyclic di-GMP signaling in bacteria: recent advances and new puzzles. J. Bacteriol. **188**(24): 8327-8334.

Ryjenkov, D. A., R. Simm, U. Romling and M. Gomelsky (2006). The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. J. Biol. Chem. **281**(41): 30310-30314.

Saxena, I. M. and R. M. Brown, Jr. (2000). Cellulose synthases and related enzymes. Curr. Opin. Plant. Biol. **3**(6): 523-531.

Saxena, I. M., R. M. Brown, Jr. and T. Dandekar (2001). Structure-function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57**(7): 1135-1148.

Saxena, I. M., R. M. Brown, Jr., M. Fevre, R. A. Geremia and B. Henrissat (1995). Multidomain architecture of beta-glycosyl transferases: implications for mechanism of action. J. Bacteriol. **177**(6): 1419-1424.

Scher, M., W. J. Lennarz and C. C. Sweeley (1968). The biosynthesis of mannosyl-1-phosphoryl-polyisoprenol in *Micrococcus lysodeikticus* and its role in mannan synthesis. Proc. Natl. Acad. Sci. USA **59**(4): 1313-1320.

Schultz, J., F. Milpetz, P. Bork and C. P. Ponting (1998). SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA **95**(11): 5857-5864.

Sinnott, M. L. (1990). Catalytic mechanisms of enzymatic glycosyl transfer. Chem. Rev. **90**: 1171-1202.

Sutherland, I. W. (1982). Biosynthesis of microbial exopolysaccharides. Adv. Microb. Physiol. **23**: 79-150.

Tarbouriech, N., S. J. Charnock and G. J. Davies (2001). Three-dimensional structures of the Mn and Mg dTDP complexes of the family GT-2 glycosyltransferase SpsA: a comparison with related NDP-sugar glycosyltransferases. J. Mol. Biol. **314**(4): 655-661.

Ünligil, U. M., S. Zhou, S. Yuwaraj, M. Sarkar, H. Schachter and J. M. Rini (2000). X-ray crystal structure of rabbit N-acetylglucosaminyltransferase I: catalytic mechanism and a new protein superfamily. EMBO J. **19**(20): 5269-5280.

Wang, S. K., I. Sa'-Correia, A. Darzins and A. M. Chakrabarty (1987). Characterization of the *Pseudomonas aeruginosa* alginate (*alg*) gene region II. J. Gen. Microbiol. **133**(8): 2303-2314.

Wiggins, C. A. and S. Munro (1998). Activity of the yeast MNN1 alpha-1,3-mannosyltransferase requires a motif conserved in many other families of glycosyltransferases. Proc. Natl. Acad. Sci. USA **95**(14): 7945-7950.

Wood, L. F., A. J. Leech and D. E. Ohman (2006). Cell wall-inhibitory antibiotics activate the alginate biosynthesis operon in *Pseudomonas aeruginosa*: roles of sigma (AlgT) and the AlgW and Prc proteases. Mol. Microbiol. **62**(2): 412-426.

Wood, L. F. and D. E. Ohman (2006). Independent regulation of MucD, an HtrA-like protease in *Pseudomonas aeruginosa*, and the role of its proteolytic motif in alginate gene regulation. J. Bacteriol. **188**(8): 3134-3137.

Woodcock, S., J. P. Mornon and B. Henrissat (1992). Detection of secondary structure elements in proteins by hydrophobic cluster analysis. Protein Eng. **5**(7): 629-635.

Wyckoff, T. J., B. Thomas, D. J. Hassett and D. J. Wozniak (2002). Static growth of mucoid *Pseudomonas aeruginosa* selects for non-mucoid variants that have acquired flagellum-dependent motility. Microbiology **148**(Pt 11): 3423-3430.