Alginate Production: Precursor Biosynthesis, Polymerization and Secretion

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Contents

Abstract The activated intracellular precursor of alginate biosynthesis is GDP-mannuronic acid. Carbon sources are oxidized to acetyl-CoA, which enters the citric acid cycle, providing the intermediate oxaloacetate. Oxaloacetate is converted via gluconeogenesis into fructose 6-phosphate. The central metabolite fructose 6-phosphate derived from gluconeogenesis is then converted to the activated alginate precursor GDP-mannuronic acid employing alginate-specific biosynthesis enzymes. This conversion requires four enzymatic steps catalysed by bifunctional phosphomannose isomerase:GDP-mannose pyrophosphorylase, phosphomannomutase and GDP-mannose dehydrogenase. GDP-mannuronic acid is polymerized to alginate by a membrane-anchored glycosyltransferase which is presumably represented by Alg8, which has been suggested to be a subunit of a multiprotein complex spanning the cytoplasmic membrane (Alg44), the periplasm (AlgX, AlgK, AlgG, AlgL) and the outer membrane (AlgE). These periplasmic proteins have been proposed to form

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a scaffold guiding the nascent alginate chain through the periplasm to the alginate-specific channel protein AlgE in the outer membrane. AlgE has been suggested to facilitate export of alginate through the outer membrane. The extended periplasmic C terminus of membrane-anchored Alg44 shows similarities to membrane fusion proteins and might colocalize the alginate polymerase (Alg8) with the export protein AlgE. The cytoplasmic N-terminal loop of Alg44 comprises a PilZ domain required for binding of the secondary messenger bis-(3′–5′)-cyclic dimeric guanosine monophosphate, the binding of which was found to be required for alginate production

1 Introduction

Alginates represent a family of non-repeating unbranched exopolysaccharides composed of various amounts of (1–4)-linked β-D-mannuronic acid and its C5-epimer α-L-guluronic acid. These sugar acids are distributed in blocks of continuous β-D-mannuronic acid residues (M-blocks), α-L-guluronic acid residues (G-blocks), or as alternating residues (MG-blocks) (Rehm and Valla 1997; Rehm 2005b). Alginates are synthesized by brown seaweeds and by bacteria belonging to the genera *Pseudomonas* and *Azotobacter* (Rehm 2002). The distribution of comonomer residues and in particular the presence of G-blocks were found to be similar in algal alginates and in alginates derived from *Azotobacter vinelandii*, whereas alginates from pseudomonads are different in that they lack G-blocks (Skjak-Braek et al. 1986). These structural differences lead to different material properties reflecting the different biological functions of the alginates (see "Material properties of alginates" by Donati and Paoletti, this volume). In brown algae and *Azotobacter* cysts (a dormant stage) alginate serves as a cell wall constituent, whereas in *Pseudomonas* it contributes to the biofilm matrix. In the opportunistic human pathogen *Pseudomonas aeruginosa* alginate is considered a virulence factor contributing to the formation of persistent biofilms after infection of the lungs of cystic fibrosis (CF) patients (Kobayashi 2005). Knowledge of alginate biosynthesis is most advanced in *P. aeruginosa* and hence in this chapter I will focus on this bacterium.

The cytosolic biosynthesis steps leading to the formation of the activated precursor GDP-mannuronic acid have been extensively investigated and are well understood; however, the actual polymerization and secretion of alginate is poorly understood. Recent studies suggested the formation of a multiprotein complex spanning the cytoplasmic membrane, the periplasm as well as the outer membrane (Jain and Ohman 2005; Oglesby et al. 2008; Remminghorst and Rehm 2006b, c).

Future research dedicated to unravelling these final steps of alginate production will be motivated by medical interest with respect to the design of alginate biosynthesis inhibitors as well as by biotechnological applications implementing the engineering of improved bacterial production organisms. Bacterial fermentation using production organisms obtained by metabolic engineering approaches should enable the biotechnological production of a wide range of defined alginates suitable for high-value applications as biomaterials in medicine (Rehm 2005; Rehm 2009). The unique material properties of alginates have already led to a variety of industrial applications, such

as stabilizing, thickening and gelling agents in food production and immobilization of cells in pharmaceutical and biotechnology industries (Paul et al. 1986). Commercial alginates are currently exclusively produced from brown seaweeds.

2 Alginate Precursor Biosynthesis

The alginate precursor GDP-mannuronic acid is synthesized in the cytosol by stepwise conversion of the central metabolite fructose 6-phosphate (Fig. 1). Three alginate-specific enzymes catalyse the four biosynthesis steps and these enzymes

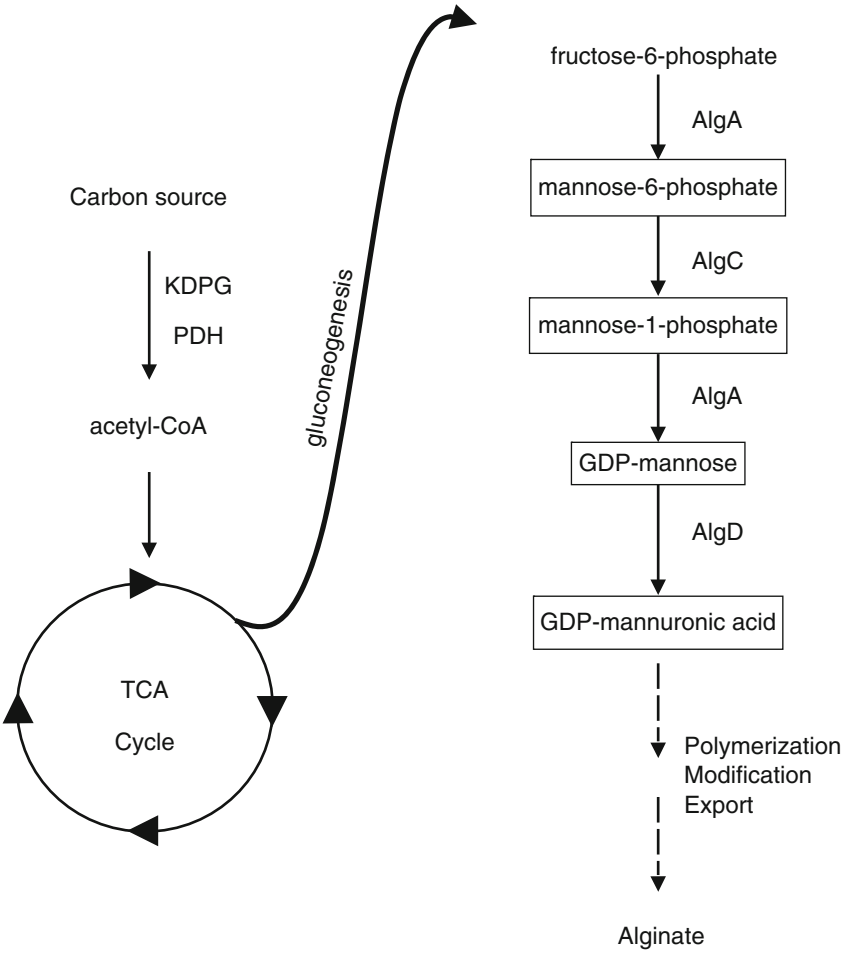

Fig. 1 Biosynthesis route of alginate in bacteria. *KDPG* ketodeoxyphosphogluconate pathway (Entner–Doudoroff pathway), *PDH* pyruvate dehydrogenase, *AlgA* phosphomannose isomerase–GDP-mannose pyrophosphorylase, *AlgC* phosphor-mannomutase, *AlgD* GDP-mannose dehydrogenase. *Boxed* intermediates are precursors of alginate. *Dashed arrows* indicate unknown biosynthesis steps

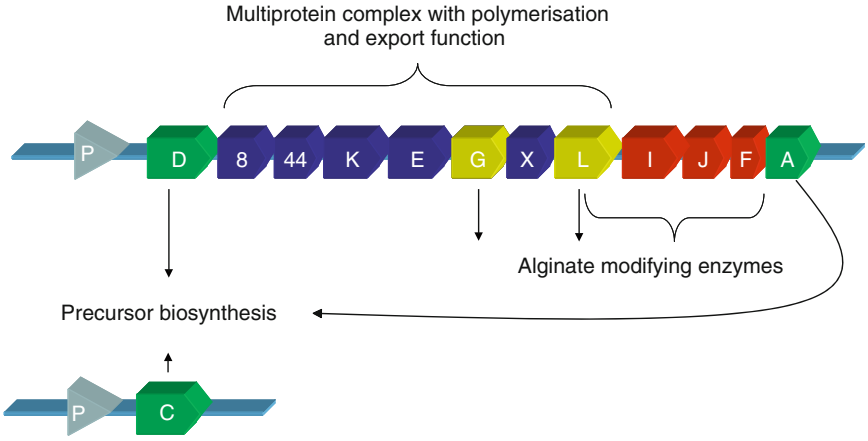

Fig. 2 The alginate biosynthesis operon and functional assignment of the genes

have been extensively characterized. Two of the genes (*algD*, *algA*) encoding these enzymes are localized in the alginate biosynthesis gene cluster and one gene (*algC*) is not colocalized (Fig. 2).

2.1 Alginate Biosynthesis Genes

The first alginate biosynthesis genes were discovered in *P. aeruginosa* motivated by the medical significance of this bacterium as an opportunistic human pathogen which often establishes chronic infections in the lung of CF patients (May et al. 1991). Alginate as a virulence factor contributes to the establishment of persistent biofilms after infection (Gacesa 1998; Russell and Gacesa 1988).

The alginate biosynthesis genes and their function have been compiled in a recent review (Rehm 2002). Seminal research by Darzins and Chakrabarty (1984) created the foundation for the identification and analysis of alginate biosynthesis genes in *P. aeruginosa*. The whole genome sequence of *P. aeruginosa* is now available and has already enabled functional genomic studies with respect to alginate biosynthesis (Lizewski et al. 2004; Stover et al. 2000). At least 24 genes were found to be directly involved in alginate biosynthesis in *P. aeruginosa*, with a few genes encoding proteins not exclusively involved in alginate biosynthesis (Table 1) (Gaona et al. 2004; Goldberg et al. 1993; Ledgham et al. 2003; Lizewski et al. 2004; Olvera et al. 1999; Wu et al. 2004; Ye et al. 1994). Twelve biosynthesis genes are colocalized in the alginate biosynthesis gene cluster, the transcription of which is regulated by one promoter upstream of the *algD* gene (Fig. 2) (Chitnis and Ohman 1993). How alginate biosynthesis gene transcription is regulated is discussed in "Alginate gene regulation" by Ohman in this volume.

Table 1 The alginate biosynthesis genes from *Pseudomonas aeruginosa*

Gene	Gene product/function or putative function[a]
algD	GDP-mannose dehydrogenase
Alg8	Glycosyltransferase/polymerase-export function?
Alg44	c-di-GMP binding-activation/membrane fusion protein?
algK	Periplasmic protein/multiprotein complex assembly
algE	Outer-membrane porin/alginate export?
algG	Mannuronan C-5-epimerase/biosynthesis?
algX	Periplasmic protein with high sequence similarity to *algJ*/scaffold protein sequestering MucD
algL	Alginate lyase/biosynthesis?
PA1167	Alginate lyase (polyguluronate lyase)/biosynthesis?
algI	O-Acetylation
algJ	O-Acetylation
algF	O-Acetylation
algA	Phosphomannose isomerase/GDP-mannose pyrophosphorylase
algB	Member of *ntrC* subclass of two-component transcriptional regulators (cognate sensor kinase is KinB)
algC	Phosphomannomutase
algH	Unknown function
algR	Regulatory component of two-component signal transduction system (cognate sensor kinase is FimS)
algQ	Histone-like transcriptional regulator binds to *algD* promoter
algP	Histone-like transcriptional regulator binds to *algD* promoter
algZ	AlgR cognate sensor (alginate and motility regulator)
algU	Homologous to *Escherichia coli* σ^E global stress response factor
mucA	Anti σ factor
mucB	Anti σ factor?
mucC	Regulator?
mucD	Homologous to *E. coli* serine protease DegP
algW	Homologous to *E. coli* serine protease DegS
mucP	Homologous to *E. coli* RseP protease involved in activation of AlgU via regulated intramembrane proteolysis cascade
mucE	Periplasmic or outer-membrane protein involved in activation of AlgU via regulated intramembrane proteolysis cascade
mucR	Alginate specific c-di-GMP synthesizing enzyme

Modified according to Rehm (2002)
c-di-GMP bis-(3′–5′)-cyclic dimeric guanosine monophosphate
[a]Putative function indicated by ?

The alginate biosynthesis genes encoding enzymes catalysing the synthesis of the precursor GDP-mannuronic acid from fructose 6-phosphate have all been functionally assigned and characterized (Table 1, Fig. 1) (Rehm and Valla 1997).

2.2 GDP-Mannuronic Acid Biosynthesis

Alginate biosynthesis enzyme activities were initially assessed in the brown alga *Fucus gardneri* by Lin and Hassid (1966) and 10 years later in *A. vinelandii* (Pindar and Bucke 1975). The central metabolite fructose 6-phosphate derived

from gluconeogenesis was found to be a precursor for the synthesis of GDP-mannuronic acid. The alginate-specific and bifunctional enzyme phosphomannose isomerase (PMI)/GDP-mannose pyrophosphorylase (GMP), encoded by the *algA* gene, catalyses the initial and third steps of GDP-mannuronic acid synthesis starting from fructose 6-phosphate (Fig. 1) (May et al. 1994). The PMI-catalysed reaction channels the fructose 6-phosphate towards alginate biosynthesis via the formation of mannose 6-phosphate (Shinabarger et al. 1991). Mannose 6-phosphate is then converted to mannose 1-phosphate by the phosphomannomutase (AlgC) (Zielinski et al. 1991). The AlgC enzyme has been found to show additionally phosphoglucomutase activity and its role in rhamnolipid and lipopolysaccharide biosynthesis was demonstrated experimentally (Goldberg et al. 1993; Olvera et al. 1999; Ye et al. 1994). The GMP activity of AlgA then converts the mannose 1-phosphate to GDP-mannose, with concomitant hydrolysis of GTP (Shinabarger et al. 1991). AlgA has been studied in more detail and amino acids required for the GMP enzyme activity were identified through site-directed mutagenesis of the *algA* gene (May et al. 1994). Replacement of Lys-175 by arginine, glutamine or glutamate resulted in a 470–3,200-fold increased K_m for mannose 1-phosphate when compared with the wild-type enzyme. These results suggested a role of Lys-175 in the binding of the substrate mannose 1-phosphate. Replacement of Arg-19 by glutamine, histidine or leucine resulted in a fourfold to sevenfold increased K_m for GTP when compared with the wild-type enzyme. Hence, Arg-19 might be involved in the binding of GTP. Limited proteolysis analysis showed that the C terminus is essential for PMI activity but not for GMP activity, suggesting that the bifunctional PMI/GMP protein is composed of two independent enzymatic domains (May et al. 1994).

GDP-mannose dehydrogenase (GMD), encoded by the *algD* gene, is a key enzyme in the biosynthesis of alginate and catalyses the almost irreversible oxidation of GDP-mannose to GDP-mannuronic acid. This is the committed step in alginate biosynthesis and it was shown to represent the metabolic bottleneck in alginate-overproducing mucoid strains of *P. aeruginosa* (Tatnell et al. 1993, 1994). Thus, AlgD has been conceived as a potential target to inhibit alginate production and hence to combat *P. aeruginosa* infections. GMD inhibitors have been identified and GMD inhibition has been shown to increase the susceptibility of a mucoid strain of *P. aeruginosa* to tobramycin, which is widely used for the treatment of CF lung infections (Snook et al. 2003). Thus, GMD inhibitors can be conceived as potential drugs which impair alginate production and the formation of characteristic biofilms and thereby strongly aid the efficiency of antibiotics. The absence of enzymes corresponding to AlgD in humans makes these inhibitors very specific by presumably avoiding severe side effects. GMD belongs to a small family of NAD^+-dependent four-electron-transfer dehydrogenases, which include UDP-glucose dehydrogenase (UGD), histidinol dehydrogenase and 3-hydroxy-3-methylglutaryl-CoA reductase. UGD and GMD have been proposed to share a similar reaction mechanism by using a single active site to catalyse the two-step conversion of an alcohol to the respective acid via a thiohemiacetal intermediate. The structure of UGD from *Streptococcus pyogenes* has been resolved and showed that the enzyme forms a dimer (Campbell et al. 2000). Biochemical characterization of the *P. aeruginosa* GMD revealed

an allosteric and cooperative behaviour, which suggested that the enzyme at least forms an oligomer composed of six subunits (Naught et al. 2002; Roychoudhury et al. 1989). In a more recent study, the crystal structure of the *P. aeruginosa* GMD in complex with its cofactor NAD(H) and reaction product, GDP-mannuronic acid, was determined at a resolution of 1.55 Å (Snook et al. 2003). The crystal structure was used to shed light on the multistep reaction catalysed by GMD. The reaction comprises four steps: (1) oxidation of a hydroxyl to an aldehyde; (2) nucleophilic attack by a thiol to form the thiohemiacetal intermediate; (3) oxidation of the intermediate to a thioester; (4) release of the product by hydrolysis. On the basis of amino acid sequence alignments and a structural comparison with UGD, the cysteine at position 268 has been proposed as the active-site nucleophilic thiol (Campbell et al. 2000; Roychoudhury et al. 1992). The significance of this residue was confirmed by the sensitivity of GMD to thioreactive agents (Shankar et al. 1995). Accordingly, the crystal structure of GMD showed that the Cys-268 thiol group and the oxygens of the carboxylate group of the mannuronic acid could be within 2.4 Å. Thus, the thiol group as a potent nucleophile presumably serves as the active site required for formation of the proposed thiohemiacetal intermediate.

3 Alginate Polymerization and Secretion

The polymerization of exopolysaccharides usually requires the activity of membrane-bound glycosyltransferases which catalyse the transfer of an activated sugar moiety onto a receptor molecule while forming a glycosidic bond. The Wz-dependent capsular polysaccharide polymerization/secretion pathways in *Escherichia coli* have been extensively studied and serve as a model for the biosynthesis of various bacterial exopolysacharides (Whitfield 2006). Repeating sugar units are synthesized attached to a lipid carrier by glycosyltransferases at the cytoplasmic membrane. The lipid carrier linked repeating unit is then transferred across the cytoplasmic membrane and polymerization occurs at the periplasmic side of the membrane. The polymerized repeating units are subsequently secreted through specific pores in the outer membrane (Whitfield 2006). However, alginate represents a non-repeating exopolysaccharide and no alginate-related lipid carrier intermediate could be identified so far. Hence, alginate polymerization and transfer across the membrane might be based on a different molecular mechanism. The polymerization and secretion of alginate are barely understood and extensive research will be required to shed light on these molecular processes (Rehm and Valla 1997; Rehm 2005).

3.1 Alginate Polymerization

The polymerization of mannuronic acid residues to alginate could resemble the lipid carrier independent cellulose synthesis in bacteria such as *Gluconacetobacter xylinus* (Ross et al. 1991). The cellulose synthase comprises a multiprotein complex of 420

kDa which resides in the cytoplasmic membrane and catalyses the processive polymerization of glucose by using UDP-glucose as a substrate. New findings related to bacterial cellulose biosynthesis were recently summarized by Valla et al. (2009).

The protein Alg8, presumably encoding a glycosyltransferase, and Alg44 were predicted to be transmembrane proteins as well as subunits of the alginate polymerase (Oglesby et al. 2008; Remminghorst and Rehm 2006b, c). Recently, Alg8 was identified as a key membrane protein for the production of alginate and multiple copies of Alg8 resulted in significant overproduction of alginate (Remminghorst and Rehm 2006c). The same study showed for the first time the in vitro synthesis of alginate by using an envelope fraction of *P. aeruginosa*. Interestingly, separation of cytoplasmic membrane and outer membrane abolished alginate polymerization activity, which strongly suggested a coordinated polymerization and secretion as well as the requirement of a multiprotein complex spanning the cytoplasmic membrane, the periplasm and the outer membrane (Fig. 3) (Remminghorst and Rehm 2006c). Additionally, the isogenic *alg8* deletion mutant did not secrete uronic acids, which were proposed to be derived from alginate lyase (AlgL) mediated degradation of misguided and unprotected alginate when found to be produced by isogenic *algK*, *algX* and *algG* (epimerase gene) deletion mutants, respectively. Interestingly, an AlgL gene deletion mutant of *P. aeruginosa* showed a swollen periplasm shortly after induction of alginate production, which suggested that AlgL does not only degrade misguided alginate but also contributes to the proposed protein scaffold (Fig. 3) (Jain and Ohman 2005). In contrast to these proposed scaffold-forming proteins (AlgK, AlgX, AlgG, AlgL), which were proposed to play a role in guidance

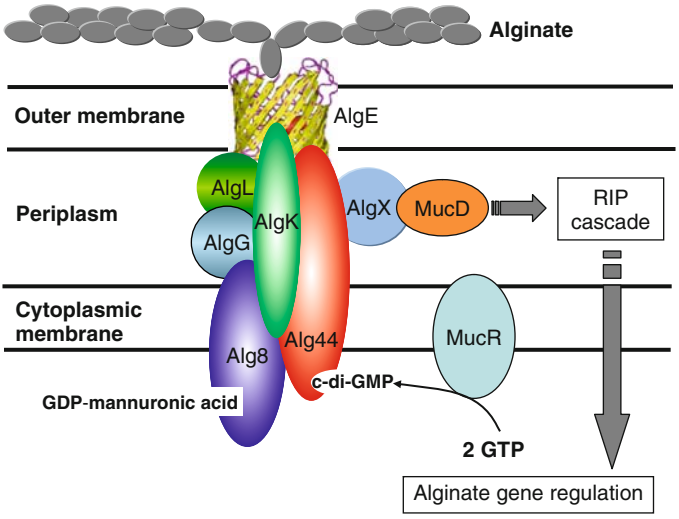

Fig. 3 Model of the proposed multiprotein complex involved in alginate polymerization/export. *c-di-GMP* bis-(3′–5′)-cyclic dimeric guanosine monophosphate, *RIP* regulated intramembrane proteolysis, *Alg* alginate gene (for a description see Table 1)

and protection of the nascent alginate chain, Alg8 seems to be directly involved in alginate polymerization. Hydrophobic cluster analysis of Alg8 indicated similarities to processive β-glycosyltransferases (e.g. cellulose or chitin synthase) (Saxena et al. 1995), which are transmembrane proteins that catalyse the formation of polysaccharide or oligosaccharide chains by transferring the sugar residue from an activated donor substrate to a growing acceptor molecule. These β-glycosyltransferases are related on the basis of their sequence pattern and especially with respect to the presence of conserved motifs and catalytic residues (Saxena et al. 2001). 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 (Ünligil et al. 2000). A threading model of Alg8 was developed on the basis of the crystal structure of SpsA, a glycosyltransferase involved in spore coat polysaccharide formation of *Bacillus subtilis* (Charnock and Davies 1999; Remminghorst and Rehm 2006c). In a recent study, a refined model for the membrane topology of Alg8 was developed using PhoA (alkaline phosphatase) and LacZ (β-glactosidase) reporter enzyme fusions, respectively (Oglesby et al. 2008; Remminghorst et al. 2009). Evidence for a large cytoplasmic loop containing the active domains and five transmembrane domains as well as for a short periplasmic loop was obtained. The presence of a cytoplasmic loop was further confirmed by successful production of only the cytoplasmic domain as soluble protein (Remminghorst et al. 2009). The C-terminal transmembrane domain of Alg8 was found to be essential for the in vivo polymerization reaction. Conserved amino acid residues of Alg8 were subjected to site-specific mutagenesis. The predicted active-site residues in the D133, D188-x-D190, L336xxR339W340 motif as well as in D295/D296 and K297 were found to be required for in vivo polymerization activity (Oglesby et al. 2008; Remminghorst et al. 2009).

Alg44 was recently demonstrated to be a membrane protein with a periplasmic C terminus and was found to be essential for alginate polymerization (Merighi et al. 2007; Remminghorst and Rehm 2006b). A refined membrane topology model of Alg44 was obtained by constructing further PhoA fusions which showed a central transmembrane domain (amino acid residues 159–177) preceded by an N-terminal transmembrane domain (amino acid residues 1–30) presumably serving as a membrane anchor (Oglesby et al. 2008). The N-terminal cytoplasmic loop comprises the PilZ domain, which is required for binding of the secondary messenger bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Fig. 3). The PilZ domain in Alg44 is essential for alginate biosynthesis, which was recently verified experimentally by assessing the capability of modified and truncated Alg44 to restore alginate biosynthesis in an *alg44*-negative mutant of *P. aeruginosa* (Merighi et al. 2007). Thus, similar to bacterial cellulose synthesis, c-di-GMP has been verified as an important activator of bacterial alginate biosynthesis which adds an additional posttranslational regulatory layer to the already complex regulatory network which controls alginate gene expression. Interestingly, more than 33 enzymes (diguanylate cyclases, phosphodiesterases) have been found to be involved in the control of intracellular levels of c-di-GMP in *P. aeruginosa* (Kulasakara et al. 2006). It has been proposed that these enzymes contrib-

ute to the formation of localized c-di-GMP pools mediating differential regulation of various responses, such as flagella biogenesis and exopolysaccharide biosynthesis. To identify the diguanylate cyclase which provides a c-di-GMP pool for Alg44 and hence for activation of alginate, a putatively membrane anchored diguanylate cyclase PA1727 (designated MucR) which also comprises a sensor domain was analysed in more detail. An isogenic knockout of this gene caused a 38-fold decrease in alginate production in *P. aeruginosa*, which suggested that this diguanylate cyclase catalyses c-di-GMP formation required for activation of alginate biosynthesis (Hay et al., 2009). The extended periplasmic C terminus of Alg44 shows similarities to membrane fusion proteins such as MexA, a membrane-bridging protein involved in the multidrug efflux system of *P. aeruginosa* (Akama et al. 2004). Hence, Alg44 could function as a subunit of a periplasmic protein scaffold and/or link Alg8 in the cytoplasmic membrane with the outer membrane protein AlgE (Remminghorst and Rehm 2006b). Experimental evidence for a function of the C terminus of Alg44 in colocalization and stabilization of a protein complex comprising Alg8 in the cytoplasmic membrane and AlgE in the outer membrane was obtained by detecting reduced levels of AlgE in an *alg44*-negative mutant (Oglesby et al. 2008). A C-terminal deletion of only 24 amino acid residues of Alg44 did not enable restoration of alginate production, further suggesting that Alg44 might act as a copolymerase essential for alginate polymerization.

AlgK and AlgX are periplasmic proteins and the respective deletion mutants showed secretion of free uronic acids presumably owing to AlgL-catalysed degradation of misguided periplasmic alginate (Gutsche et al. 2006; Jain and Ohman, 1998; Jain and Ohman 2005).

A pull-down experiment with Strep-tag-labelled AlgX produced in an isogenic *algX*-negative mutant of *P. aeruginosa* resulted in copurification of MucD, which suggested that AlgX specifically interacts with the serine protease MucD (Gutsche et al. 2006). However, no evidence for interaction with the other proposed subunits of the alginate polymerase/secretion complex could be obtained (Gutsche et al. 2006). Since knockout mutants of the *mucD* gene resulted in a mucoid phenotype, MucD was proposed as a negative regulator of alginate biosynthesis (Boucher et al. 1996; Wood and Ohman 2006; Yorgey et al. 2001). The negative regulator function was recently shown to require the serine protease activity (Wood and Ohman 2006). AlgX as a subunit of the alginate polymerase/secretion complex might sequester MucD in the periplasm and thereby interfere with its negative regulatory function. In a recent study, a regulated intramembrane proteolysis (RIP) cascade in the envelope of *P. aeruginosa* was proposed that ultimately leads to degradation of membrane-anchored MucA. Degradation of MucA causes the release of sequestered alginate-specific sigma-factor AlgU, which then enables transcription of the alginate biosynthesis genes (Qiu et al. 2007) (see "Alginate gene regulation" by Ohman, this volume, for further details). In the absence of AlgX, MucD could interfere with RIP activation of alginate gene transcription by degrading proteins such as AlgW, MucP, MucE and MucB (Fig. 3). These proteins have been proposed to be involved in the RIP cascade (Qiu et al. 2007).

The AlgK sequence showed an apparent signal peptide characteristic of a lipoprotein. AlgK-PhoA as well as AlgK-β-lactamase fusion proteins were found to show reporter enzyme activity, which suggested a periplasmic subcellular localization while they were probably anchored in the cytoplasmic membrane (Aarons et al. 1997; Jain and Ohman 1998). Overall the amino acid sequence of AlgK does not indicate a specific function of the protein in alginate polymerization/secretion. However, the AlgK sequence shows four Sel1-like repeats (SLR) which are characteristic of proteins forming modular architectures and which all seem to serve as adaptor proteins for the assembly of macromolecular complexes (Mittl and Schneider-Brachert 2007). Thus, AlgK might play a key role in the assembly of the alginate polymerization/secretion multiprotein complex. Recently, AlgK was subjected to a structural analysis in an attempt to verify the presence of the putative SLR motifs and provide insight into the function of AlgK. AlgK could be crystallized and preliminary X-ray data were obtained (Keiski et al. 2007). On the basis of density calculations, the authors estimated that four molecules of the protein are present in the asymmetric unit (Keiski et al. 2007). Size-exclusion chromatography results using purified AlgK provided evidence that AlgK forms a dimer in solution, suggesting that two dimers constitute the asymmetric unit in crystallized AlgK.

AlgX, AlgK, Alg44, AlgG together with AlgL were proposed to form a protein scaffold in the periplasm surrounding the nascent alginate chain and guiding the polymer to AlgE in the outer membrane (Fig. 3) (Gutsche et al. 2006; Jain and Ohman 1998, 2005; Jain et al. 2003; Remminghorst and Rehm 2006a, b), while AlgL might also clear the periplasm from misguided alginate and AlgG introduces guluronic acid residues (Bakkevig et al. 2005). Interestingly, except for the AlgX-MucD interaction, none of the proposed protein-protein interactions in the alginate polymerization/secretion multiprotein complex were confirmed experimentally.

3.2 Alginate Export

An early comparative analysis of outer-membrane protein profiles from various mucoid and non-mucoid *P. aeruginosa* strains indicated the presence of a 54-kDa protein exclusively found in mucoid strains (Grabert et al. 1990); hence, it was proposed that this outer-membrane protein could play a role in alginate biosynthesis. Shortly after the detection of the mucoid-specific 54-kDa outer-membrane protein, the *algE* gene in *P. aeruginosa* was identified as an essential gene for alginate production and which is also part of the alginate biosynthesis gene cluster (Chu et al. 1991). In the same study the *algE* gene product was identified as an insoluble presumably membrane bound protein with an apparent molecular mass of 54 kDa. Purification of the 54-kDa protein after solubilization with octylglucoside enabled N-terminal sequencing which confirmed that the 54-kDa protein corresponded to AlgE (Grabert et al. 1991).

The fact that AlgE is strictly associated with the alginate-overproducing mucoid phenotype of *P. aeruginosa* triggered a study investigating the immunogenicity of

AlgE as well as the use AlgE as an antigen for antibody capture in diagnostic applications (Rehm et al. 1994b). Purified and native AlgE was injected into rabbits and a strong antibody response 9 weeks after injection suggested a strong immunogenicity of AlgE (Rehm et al. 1994b). Hence, it was conceived that AlgE could be used for antibody detection in sera from CF patients. An antibody-capture ELISA with AlgE as an antigen was established and the analysis of 41 sera from CF patient showed a strong correlation with the infection status of the CF patients (Rehm et al. 1994b). None of the 23 control sera from healthy humans showed significant levels of anti-AlgE antibodies. Interestingly, CF patients who became infected only a few weeks before the blood samples were taken already showed a significant anti-AlgE antibody titre, suggesting the conversion to the mucoid form occurs early after infection with non-mucoid forms (Rehm et al. 1994b). Since CF patients are initially infected by non-mucoid *P. aeruginosa* and the mucoid form, which correlates with the establishment of a chronic infection, emerges in the CF lung during infection, the AlgE-based ELISA might represent an important differential diagnostic tool to assess the infection status. The infection status significantly informs the antibacterial treatment regimen, because mucoid *P. aeruginosa* shows a different and mainly increased antibiotic resistance, when compared with the non-mucoid variant. The strong AlgE immunogenicity also suggested an application of this mucoid-specific outer-membrane protein as part of a multicomponent experimental vaccine which could mediate protection against colonization with mucoid forms of *P. aeruginosa* (Rehm et al. 1994b).

AlgE is only represented as a minor protein with a low copy number in the outer membrane. To produce significant amounts of AlgE for functional analysis, *algE* was overexpressed in *E. coli* using a T7-promoter-based overexpression system. Recombinant and mature AlgE was localized to the outer membrane of *E. coli* by using immunoblotting analysis. This analysis suggested that the signal peptide was properly cleaved off during the secretion process, while traces of unprocessed AlgE were still detectable in the cytoplasmic membrane (Rehm et al. 1994a). Recombinant AlgE was solubilized from the *E. coli* outer membrane and purified by immobilized metal ion affinity chromatography followed by anion-exchange chromatography. Outer-membrane proteins have been described to function as general or specific porins, which form a β-barrel and facilitate the transport of various or specific compounds through the outer membrane (Buchanan 1999; Delcour 2002; Schulz 1996). Thus, to assess whether AlgE functions as such a channel protein it was subjected to electrophysiological analysis using planar lipid bilayers. These experiments demonstrated that AlgE can spontaneously incorporate into planar lipid bilayers while causing single-channel current fluctuations with a low mean conductance of 0.76 nS and a very short mean open-state lifetime of 0.7 ms (Rehm et al. 1994a). The electrophysiological analysis suggested that AlgE forms a channel protein in the outer membrane with unusual properties hitherto not described for other outer-membrane channel proteins (Delcour 2002). The AlgE channel was characterized in more detail by using differently sized cations and anions, respectively. Only the size of the anion had an impact on the mean single-channel conductance and increasing anion size lowered the mean single-channel conductance. These findings suggested that AlgE

forms an anion-specific channel which could facilitate the export of the anionic alginate through the outer membrane. This proposed function of AlgE was further supported by the finding that GDP-mannuronic acid can partially block the AlgE channel (Rehm et al. 1994a). Topological models of AlgE and its homologue AlgJ from *A. vinelandii* have been developed on the basis of secondary structure predictions, hydrophilicity analysis and known outer membrane protein structures, resulting in the proposal of a β-barrel consisting of 18 β-strands (Fig. 3) (Rehm et al. 1994a; Rehm 1996). Recently, homology modelling was used to generate a three-dimensional model of AlgE suggesting a pore diameter suitable for alginate export (Rehm 2002). These data supported the hypothesis that AlgE forms an alginate-specific pore which enables export of the nascent alginate chain through the outer membrane. The AlgE protein is the only outer-membrane protein which has been found to be required for alginate biosynthesis and which might be colocalized with the alginate polymerase by the membrane fusion protein function of Alg44.

4 Conclusion and Future Perspectives

Although the alginate biosynthesis gene cluster was identified in the late 1980s, the functional assignment of various genes presumably contributing to alginate polymerization/secretion is still at an early stage. Except for the AlgX–MucD interaction, none of the proposed protein-protein interactions required to form an envelope-spanning multiprotein complex have been confirmed experimentally. Further extensive research will be required to shed light on the molecular mechanisms underlying alginate polymerization and secretion as well as the c-di-GMP-mediated activation. The understanding of the final steps in alginate production might inform the design of specific inhibitors when considering the role of alginate as a virulence factor and/or it might help to overcome the production bottleneck when considering alginate as an important biopolymer for medical and biotechnological applications. Although commercial production of alginate relies almost entirely on cheap algal sources, the ability to engineer bacterial alginates will make continuous fermentative production using bacteria increasingly attractive. Knowledge of how alginate composition and molecular weight contribute to material properties and knowledge of what material properties are required to meet medical specifications will provide an enormous opportunity for the use of engineered bacteria for the production of tailor-made alginates.

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