The role of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm architecture

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Aamir Ghafoor

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen. It causes chronic lung infections in the cystic fibrosis patients. These infections become highly resistant to antibacterial treatments. Bacteria develop this resistance because they become protected inside biofilms. Biofilms are microbial communities enmeshed in a partially self-produced and partially recruited, impregnable extracellular matrix. The matrix is composed of extracellular DNA, proteins, lipids and exopolysaccharides. The exopolysaccharides play an imperative role in architecture of the biofilm matrix. *P. aeruginosa* produces three distinct exopolysaccharides; Psl, Pel and alginate. In this study, non-mucoid strain PAO1 and mucoid (producing excessive alginate) strain PDO300 of *P. aeruginosa* were used to generate mutants deficient in one or more exopolysaccharides. Role of these three exopolysaccharides in biofilm formation was investigated. Results showed that the absence of alginate altered the architecture of biofilms in PDO300 as well as in PAO1, when compared to biofilms formed by the respective parent strains. Psl was found indispensable for mushroom-like shape of the biofilms in both strains. Pel was required for the compactness of the biofilms, but PAO1 formed mushroom-like structures even in the absence of Pel. However, Pel-deficient PDO300 did not form mature biofilm, suggesting differential role of Pel in the two strains. Psl-only as well as Pel-only, producing mutants were able to formed multilayer biofilm. Production of one type of exopolysaccharide appeared to influence production of the other types of exopolysaccharide. Psl-deficient mutants increased the production of Pel, while Pel-deficient mutants showed a ten-fold increase in the production of alginate. Furthermore, absence of negatively charged alginate in the biofilm was compensated by eDNA. Regulation of exopolysaccharide biosynthesis operons showed a high expression of *psl* operon in PAO1, whereas its expression in PDO300 was surprisingly low and confined to a few cells near the base. A high and uniform expression of the *algD* operon in PDO300 was observed at all times during biofilm development. A low expression of *algD* operon was also detected in PAO1. Expression of the *pel* operon was confined to the stalk of PDO300 and PAO1. The role of PelF, the only glycosyltransferase encoded by *pel* operon, in Pel biosynthesis was investigated and found to be a soluble glycosyltransferase which uses UDP-glucose towards Pel biosynthesis. Site directed mutagenesis revealed that conserved R-325 and K-330 were essential for the PelF activity.

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CHAPTER I

General Introduction

ABSTRACT

More than 90% bacteria live as a biofilm in natural habitats. This surface-attached, sessile mode of life confers many advantages to bacteria and increases their survival. *Pseudomonas aeruginosa*, an opportunistic pathogen, has been extensively studied as a model organism for biofilm studies. Biofilm formation is a stepwise process in which motile bacteria attach to a surface and initiate a cascade of changes. Consequently these bacteria become phenotypically different from their planktonic counterparts. Initially these bacteria attach reversibly to the surface via hydrophobic interactions between the bacterial surface proteins and the substratum. In response to environmental cues, intracellular concentration of a secondary messenger, c-di-GMP, increases in the bacterial cells. This increase in c-di-GMP results in loss of motility, expression of specific adhesins, and irreversible attachment of the bacteria. These bacteria start proliferating while residing in close proximity. The bacterial cells sense the presence of other community members in their close proximity, and start communicating with each other through a mechanism known as quorum sensing. These bacterial cells produce and recruit extracellular polymeric substances (EPS) which encase the bacteria and protect them from unfavourable environmental conditions, predators and biocidal compounds. Components of the EPS include eDNA, proteins, lipids and exopolysaccharides. The exopolysaccharides produced and secreted by the bacteria play a vital role in biofilm formation. *Pseudomonas aeruginosa* produces various types of exopolysaccharides, but three of them are considered very important in biofilm formation; mannose-rich Psl, glucose-rich Pel, and uronic acid-rich alginate. These three exopolysaccharides are synthesized and secreted by the protein-complex encoded by their respective operons. The role played by these exopolysaccharides in architecture and function of biofilm is currently under extensive research. This review presents the mechanism of biofilm formation and the role of exopolysaccharides in biofilm.

INTRODUCTION

For a long time in the history of microbiology, microorganisms were considered as freely suspended cells in nutritionally rich media and were characterized on the basis of their growth characteristics in the media provided. Heukelekian and Heller showed for the first time that the presence of a surface for attachment substantially enhanced the bacterial growth (1). A subsequent study demonstrated that the number of bacteria on the surface were more as compared to their surrounding environment. These indirect evidences provided clues that bacteria can grow as a community after attaching to the surface (2). This community of surface-attached bacteria is now known as biofilm. However, a detailed study of biofilm was made possible only after the use of electron microscopy. Jones *et al.* (3) used electron microscopy to show that biofilm formed in seawater on a trickling filter was composed of many different kinds of organisms. With the help of polysaccharide-specific stains, these workers also provided evidence for the presence of polysaccharides in extracellular matrix of bacterial biofilms. In subsequent years it was shown that microbial biofilms in industrial waste water were not only very persistent but also highly resistant to disinfectants such as chlorine (4). Later, a theory was proposed which elucidated the mechanism through which microorganisms attach to a surface and the advantages amassed by the microorganisms from this ecologic niche (5). In the last two decades, the use of scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and molecular biology techniques to study gene regulation have helped to increase our understanding of architecture and functioning of biofilms.

MICROBIAL BIOFILMS

Biofilm is a consortium of microbes which is irreversibly attached (not removed by gentle washing) to a surface and enclosed in a matrix of extracellular polymeric substances (EPS). The EPS constitute 50- 90% of the total organic mass of the biofilm and is composed of exopolysaccharides, extracellular DNA (eDNA), proteins, lipids and humic substances (6). The extracellular polymeric substances encased microbes cells form microcolonies which are separated from each other by the presence of water channels (7). These water channels allow liquid to flow through them which facilitates

transfer of nutrients, oxygen, waste metabolites and even antimicrobial agents into the surrounding microcolonies

It is suggested that more than 90% of bacteria live in biofilm mode (8, 9). Inside the biofilm, cells living in close proximity can have an increased rate of gene transfer, well developed cooperation and quorum sensing [reviewed in (10)]. Furthermore, compared to planktonic bacteria, biofilms can better endure the negative consequences of pH changes, nutrient scarcity, free radicals, biocides and antimicrobial agents [reviewed in (8)].

(i) Importance of microbial biofilms

Microbial biofilms impact human life in different ways. Bacterial biofilms on medical devices such as catheters, endoscope tubes, contact lenses and implanted prosthetic devices, are a known source of nosocomial infections [reviewed in (11)]. Microorganisms form biofilms on internal and external human surfaces and cause various infections including dental plaques, skin infections, urinary tract infections and chronic lung infections [reviewed in (12). Recent studies have shown that chronic lung infections in cystic fibrosis (CF) patients and some persistent wound infections are due to a biofilm forming microorganism, *Pseudomonas aeruginosa* (13-16).

Biofilm forming microbes also cause infections in animals. Otitis (both, media and externa), a chronic and 'hard to eradicate' infection, is caused by *P. aeruginosa* and *Staphylococcus intermedius* in dogs (17, 18). The persistent nature of chronic mastitis in dairy animals is attributed to the ability of the microorganisms to form biofilms [reviewed in (19)]. Biofilms in the food processing industry have also been recognized as a big problem. Bacteria can adhere and grow as biofilm in pipelines. These biofilms are difficult to eradicate due to their resistant nature, and become a continuous source of bacterial contamination [reviewed in (20)].

Microbes also form biofilms in the gastrointestinal tract of humans and animals. These biofilms provide an ecological niche where bacteria can interact with each other and with the host cells. These biofilms develop a relationship with the host which may be beneficial or harmful for the host [reviewed in (21)] .

These diverse effects of biofilms on human and animal lives described above have attracted a lot of attention by researchers. Although many different bacterial

species have been used to study the architecture, function, regulation and control of biofilms, a lot of research has been conducted using *P. aeruginosa* as a model organism. Medical relevance, ubiquity, and ability to survive in diverse environments have made *P. aeruginosa* an ideal organism for biofilm study. In this review, salient features of biofilm formation are discussed, focusing on *P. aeruginosa* as the model organism.

ii) The model organism: *Pseudomonas aeruginosa*

P. aeruginosa is a gram negative, rod shaped bacterium which has an ability to adapt to a wide range of environments. In the presence of water the bacterium can colonize both biotic and abiotic surfaces. *P. aeruginosa* is an opportunistic pathogen which can infect plants, insect, worms and mammals (22-24). A variety of virulence factors produced by *P. aeruginosa* help it to survive in wide range of hosts. In addition, *P. aeruginosa* increases the chance of its survival by living both planktonic and sessile modes of life and can switch from one mode of life to the other in response to changing environmental conditions. The ability of *P. aeruginosa* to cause acute and chronic infections in immuno-compromised patients can be linked with planktonic and sessile modes of life respectively. Both modes of life are governed by various transcriptional regulators produced by the bacterium (25). During planktonic life, motile bacteria produce numerous virulence factors which help the bacteria to cause acute infections in the host. These factors include(26): type IV pili (26, 27), the Type III secretion system (T3SS) (27), the Type II secretion system (T2SS) secreted toxins and proteases. On the other hand, *P aeruginosa* can also become non-motile and starts developing a biofilm which protects bacteria from host response and increases its chance of survival inside the host. This sessile mode of life is a major cause of chronic infections in human and animals. A better understanding of this bacterial mode of life will lead us to develop better treatment and control strategies.

BIOFILM FORMATION

Biofilm formation is a stepwise, complex and dynamic process which involves many physiological, chemical, genetic and biological processes. Biofilm formation can be roughly divided into the following steps: reversible and irreversible attachment to substratum surface, followed by microcolony and biofilm formation with the help of

EPS (produced and recruited by constituent bacterial cells), and finally dispersal of biofilm. A schematic representation of biofilm formation in *P. aeruginosa* is presented in Figure 1.

Figure 1 Schematic diagram of biofilm development. Reproduced with permission (28).

i) Attachment to the surface

Biofilm formation is initiated by attachment of free living cells to a surface. With the help of Brownian movement and flagellar-dependent motility, bacterial cells reach to an appropriate surface described below.

a) The role of substratum in attachment

Compared to growth in liquid, attachment to a surface provides several advantages to the bacterial cells. First, surfaces provide a good nutritional supply because most of the macro molecules are adsorbed to the surface (called "conditioning" of the surface) [reviewed in (10)]. Second, laminar flow velocity and shear forces are minimal just above the surface at the liquid-solid interface, which allows settlement of the bacterial cells on the surface [reviewed in (10, 29)]. Physical and chemical properties of the surface may play an important role in attachment, as rough surfaces provide an extended surface area and friction for strong attachment (30). Similarly, hydrophobicity and hydrophilicity of the surfaces also play a role in bacterial attachment. Previous studies

suggest that microorganisms readily attach to hydrophobic surfaces, such as Teflon and plastics, as compared to hydrophilic surfaces, such as glass and metals (31-33).

b) Environmental cues for attachment

Bacterial attachment to surfaces is initiated in response to some environmental cues. Scarcity of nutrition is one of the important environmental cues in response to which many bacterial species initiate attachment (8, 34, 35). Limited supply of inorganic phosphate (P_i) is sensed by the bacteria for initiation of biofilm, but a concentration of P_i below a certain threshold level impacts negatively and results in impaired attachment in *Pseudomonas aureofaciens* PA147-2 (36). In *Escherichia coli* K-12, different nutritional sources were tested and it was shown that carbon sources which were metabolized to produce acetyl coenzyme A, acetyl phosphate, and acetate resulted in initiation of biofilm formation (37). The presence of monosaccharides (the building blocks of extracellular polysaccharide) in the growth media is sensed by *Vibrio cholerae* as a cue to form biofilm (38). In addition, osmolarity, divalent cations and pH are also environmental factors which are sensed as cues by many bacterial species to initiate attachment and begin biofilm formation [reviewed in (39)].

c) Reversible attachment

Attachment can either be reversible or irreversible. The reversible attachment is a transitory phase and bacterial cells can detach from the surface at any time to become freely swimming cells again. Reversibly attached bacterial cells adhere to a surface via a single pole using a flagellum, fimbriae or other adhesins produced by the bacterium [reviewed in (39)]. This attachment is initiated when bacterial cells make first contact with the surface and overcome the repulsive electrostatic forces. A repulsion is expected, due to net negative charges both on bacterial and other surfaces [reviewed in (40, 41)]. However, these repulsive forces are overcome by the hydrophobic interactions between adhesins present at the bacterial surface and the attachment surface (42). The role of fimbriae in bacterial attachment to surfaces has been reported by many studies (43, 44) and most fimbriae have been shown to contain high numbers of hydrophobic amino acid residues and may contribute to cell surface hydrophobicity (43). Flagella have also been shown required for attachment.

In *P. aeruginosa*, mutations in flagellar biosynthesis and type IV pili genes rendered the mutants defective in attachment to the surface (45). It was speculated that

this defect in attachment was due to inability of the mutants to swim and reach the surface. However, later studies proposed that FilD, a flagellar cap protein, interacts with the mucin and may be required for attachment in the lungs of cystic fibrosis (CF) patients (46). Recent studies have shown that attachment is reduced in the absence of structural flagellar proteins and defects in flagellar motility also results in impaired attachment and reduced biofilm growth. *P. aeruginosa* have a single flagellum and one motor but two stators, MotAB and MotCD, which provide energy for flagellar rotation. Although the presence of a single stator was sufficient for flagellar movement, both were shown to be essential for attachment and biofilm development. Using beads coated with purified Type IV pili, a recent study has demonstrated that type IV pili are essential and sufficient for maximal binding to the surface of polarized epithelial host cells (47). In addition, a chaperone-usher pathway (Cup) which synthesizes and assembles the fimbriae also has been suggested as an essential component for attachment to abiotic surfaces (48).

d) Irreversible attachment

Irreversibly attached bacteria remain fixed to the surface and form a monolayer. In the case of irreversible attachment, cells are adhered to a surface along their long axes and show reduced flagellar movements [Reviewed in (39)]. A few factors have been identified in some bacterial species which are suggested to be involved in the transition from reversible to irreversible attachment state.

The transition from reversible to irreversible attachment in *P aeruginosa* has been linked with SadB/SadC/BifA protein complexes. These proteins control swarming motility and biofilm formation [reviewed in (39)]. In many bacteria transition from planktonic to sessile mode of life is controlled by a secondary messenger known as cyclic diguanylate monophosphate (c-di-GMP) which is synthesized from two molecules of GTP. The group of proteins which synthesize c-di-GMP are known as diguanylate cyclases (DGCs) and the group of proteins which are involved in the degradation of this secondary messenger are known as phosphodiesterases (PDEs) (49). In *P aeruginosa,* SadC is a DGC which synthesises c-di-GMP in a localized area and, as a result of that, motility is reduced and biofilm development is increased (50, 51). In contrast, BifA, a PDE, c-di-GMP degrading enzyme, increases motility of bacterial cells, decreases attachment and reduces biofilm development (50, 51). SadB is

suggested to control the transition from reversible to irreversible by decreasing flagellar reversal rate and increasing exopolysaccharide production (52, 53).

In *Pseudomonas fluorescens*, a large protein, LapA, is secreted which is associated with the cell surface and helps the bacterial cell to attach permanently to surfaces (54). Similarly, another large protein, LapF, secreted by *Pseudomonas putida* is required for attachment and colonization on the roots of the plants (55). Interestingly no orthologues of *LapA* and *LapF* are found in *P. aeruginosa* (56) but presence of large protein, CdrA (c-di-GMP-regulated protein A), has been suggested to be required to form stabilized biofilm structures by interacting with Psl (57). In *E. coli*, biosynthesis and secretion of poly-δ-glutamate (PGA) is proposed as an important adhesin required for permanent attachment (58). In *Caulobacter crescentus* transition is initiated by removal of flagellum with the help of protease (59) and an exopolysaccharide 'holdfast' is produced which is composed of oligomers of *N*-acetylglucosamine (60, 61). The holdfast acts as an adhesin and firmly attaches the bacterium to the surface (62).

ii) Microcolony and biofilm formation

In some microbes, transition from reversible to irreversible attachment is mediated by the presence of EPS. After attachment, the bacterial cells start proliferating to form localized microcolonies and EPS production increases within the microcolonies. Eventually a hydrated extracellular matrix encases the immobilized bacterial cells, where bacterial cell division continues inside the matrix and results in formation of a heterogeneous biofilm [reviewed in (63, 64)]. Increase in heterogeneity of biofilm allows the development of physical and chemical microgradients inside the biofilm, including those of nutrients, pH and oxygen [reviewed in (65)]. For instance, when biofilm attains a thickness of 10-25μm, the microenvironment near the attachment surface changes and becomes anaerobic (66). This decreased oxygen supply may result in altered gene expression in the cells. Many subpopulations may develop within the biofilm with differential gene expression in different subpopulations of the biofilm. For instance, a study showed that enzymatic activity of an oxygen-dependent alkaline phosphatase was high at the surface and low at the base of *P. aeruginosa* biofilm (67). This was related to less oxygen supply near the base compared to that on the surface. Similarly, protein and DNA synthesis was observed in different subpopulation of *S.*

aureus biofilm. The cells located near the surface with ample supply of oxygen showed high metabolic activity, whereas the cells close to agar were metabolically less active (68). This presence of sub-populations of cells within biofilm is an indicative of division of labour. This division of labour between cells in the biofilm also influences the architecture of the biofilm (69).

The architecture of hydrated biofilm was impossible to study using light or electron microscopy, but became evident using CLSM. Many studies have demonstrated that biofilm are composed of clustered cells, encased in an extracellular matrix. On the basis of the shape of these cell clusters, biofilm architecture can be designated as flat, filamentous, cylindrical or mushroom-like [reviewed in (70, 71)]. Architecture of the biofilm is also influenced by availability of nutrients. The wild-type *P. aeruginosa* PAO1 usually forms mushroom-like microcolonies. However, when genes required for the iron binding protein pyoverdine were inactivated, the mutants formed very thin and unstructured biofilms even in the presence of iron in the media (72). Increase in iron supply above a threshold level also proved to be harmful for microcolony formation in wild type *P. aeruginosa* PAO1 (73).

iii) Extracellular matrix formation.

EPS make a meshwork of polymers which contribute to cell to surface attachment, cell to cell attachment, and provide a scaffold for the architecture of the biofilm. The EPS hold the cells together and help them to live in close proximity where cell to cell communication is made possible; this is essential for the functioning of the biofilm. EPS protect bacterial cells from desiccation, biocidal agents, protozoal grazing, heavy metals, free radicals and the host immune system [reviewed in (70)].

The Matrix is composed of EPS, metal ions and water. Initially the extracellular polysaccharides were thought to be the only polymeric substances present in the biofilm matrix, but many other components have subsequently been identified. Components of the EPS are eDNA, proteins, lipids, polysaccharides and humic substances [reviewed in (70, 74)].

a) Extracellular DNA

Previously, eDNA was considered as a bi-product of lysed cells, but recent studies have shown that eDNA is a very important polymer in the matrix of biofilms formed by both

Gram-positive and Gram-negative bacteria (75, 76). eDNA helps to maintain the architecture of the biofilm and acts like glue to stabilize the cell to cell connections (77). A role of eDNA has also been suggested in protection of bacterial cells in biofilm against the harmful effects of cationic antibacterial polypeptides (CAP) (78). In bacterial biofilms, eDNA is discharged by various mechanisms including prophage-mediated cell death (13, 79), quorum sensing regulated DNA release (75), and by outer membrane vesicles (OMV) produced by living cells (80, 81). In *P. aeruginosa*, eDNA plays an important role in architecture and function of the biofilm (77). Treatment of *P. aeruginosa* biofilms with DNase at early stages of biofilm development dissolved the biofilm. However, DNase treatment at later stages did not prove detrimental (77). In a young two days-old *P. aeruginosa* biofilm, eDNA was located on the surface of microcolonies, whereas it was found around the stalk of mushroom-like biofilm structures in the mature biofilm (75). Recently eDNA has been shown as important component for the motility of the *P. aeruginosa* cells. It acts as trails and allows cells to move on it and this helps bacterial biofilms to organise their cells in the required way to develop the architecture of the biofilm (82).

The role of eDNA has been studied in various other bacterial biofilms. In *Haemophilus influenzae* biofilms, eDNA forms a dense meshwork of fine threads like a 'thick rope' that span water channels (83). Members of the genus *Rhodovulum* form self-flocculating, aggregating structures with the help of exopolysaccharides, proteins and eDNA. Interestingly, DNase treatment of these clumps resulted in breakdown of the structures, whereas protein and polysaccharide lysing enzymes showed no impact on biofilm architecture, suggesting eDNA as the main polymer involved in biofilm formation in *Rhodovulum* (84).

b) Proteins

Proteins are also important structural components of the extracellular matrix in biofilm. In addition, some enzymes are also held in EPS which make an external digestive system for bacterial cells.

In *P. aeruginosa*, two galactose-specific lectins, LecA and LecB, have been shown important for the maintenance of the biofilm architecture (85, 86). Biofilm was completely disrupted when LecB was targeted with high-affinity ligand (87). Role of another polymeric protein, CdrA, has also been demonstrated for *P aeruginosa* biofilm.

CdrA binds with exopolysaccharide Psl, and links Psl with Psl or Psl with cell (57). In addition, Cup fimbriae (88) and type IV pili (89) are also required for architecture of the *P. aeruginosa* biofilm. Similarly, TasA in *B. subtilis* (90) and Bap in *S. aureus* (91) are two more examples of proteins which are required to form biofilms by the respective bacteria species.

c) Exopolysaccharides

Many bacteria produce extracellular polysaccharides that play important roles in bacterial virulence. These extracellular polysaccharides can be classified into two subclasses: (a) capsular polysaccharides, which remain associated with the cells after harvesting the bacteria by centrifugation, and (b) exopolysaccharides which remain in the supernatant. This distinction can be well defined for bacteria grown in shaken cultures, but it is difficult to discriminate between the two when studying structured communities such as biofilms. Nevertheless, many studies strongly support the notion that biofilm architecture is mainly defined by these extracellular polysaccharides produced by the biofilm-forming bacterial species [reviewed in (74)].

Cellulose is one of the most studied exopolysaccharide regarding biofilm formation. *Gluconacetobacter xylinus* is the first species shown to produce extracellular cellulose (92). Cellulose has also been reported in some other bacterial species, for instance *Salmonella typhimurium* and *Escherichia coli*. (93). Comparative genome analysis of many other species has revealed the presence of genes homologous to the *G. xylinus bcs* (bacterial cellulose synthesis), indicating their capability to produce cellulose (94). Cellulose is shown to be involved in biofilm formation in such bacterial species. It forms gel which retains water and consequently protects the residing bacteria from desiccation (95).

In addition to cellulose, there are other exopolysaccharides known to be involved in the formation of extracellular matrices. For example, the exopolysaccharide intercellular adhesin (PIA), produced by enzymes encoded by the *ica* locus in staphylococcal species (96). PIA synthesized by *Staphylococcus epidermidis* and *S. aureus* is also known as poly-N-acetyl glucosamine (PNAG) polymer. These polymers act as adhesins, and are essential for biofilm architecture (97-99). PIA-like polymers were also reported to be synthesized in other bacterial species. For example in *E. coli* MG1655, a polymer similar to PIA has been shown to play a role in biofilm

formation (100). Similarly, in *Yersinia pestis*, synthesizes a polymer similar to PIA which is required for the biofilm formation inside the mouth of parasite host which results in an irritation in parasite and the parasite bites to the human host. Consequently results in spreading of plague (101). Colanic acid is another sugar polymer produced by *E. coli*, composed of galactose, fructose and glucose, and commonly found in extracellular matrix of biofilms formed by *E coli* (102).

Most strains of the Gram-positive bacterium *B. subtilis* are capable of synthesizing two polymers: the exopolysaccharides, EPS[1] and PGA, which play important role in the process of biofilm formation (90, 103). The role played by each polymer depends on the strain and environment surrounding the biofilm. For instance, the wild strain NCIB3610 needs exopolysaccharide EPS[1] but not PGA to form the biofilm (90). PGA overproducing mutants formed mucoid colonies and biofilm which lost its structure. In some wild strains like RO-FF-1 and the laboratory strain JH642, PGA is naturally produced and is crucial for surface-attached biofilm formation (103).

Teichoic acid is another exopolysaccharide which constitutes the biofilm matrix in *S. epidermidis* (104). *S. epidermidis* synthesizes two types of teichoic acid: one which is linked with the bacterial membrane, and the other which is secreted into extracellular milieu. Secretion of the extracellular teichoic acid increases viscosity of the colony [reviewed in (105)].

P. aeruginosa has been extensively used as a model organism to study biofilms. Three exopolysaccharides important in biofilm formation in *P. aeruginosa* are Psl, Pel and alginate. Psl is a mannose-rich exopolysaccharide, which has been shown essential for the initial attachment and biofilm architecture (106). The Role of Pel, likely to be glucose-rich exopolysaccharide (107), is not well understood yet. Alginate is an anionic polymer of manuronic acid and gluronic acid and plays a very important role in biofilm architecture and function (108).

All above mentioned polysaccharides play key role in biofilm formation. A detailed understanding of these exopolysaccharides will help us to understand the 'building blocks' of biofilm. A detailed account of the exopolysaccharides produced by

1 Here, EPS is a name of the exopolysaccharide synthesized by *Bacillus subtilis* genes, *eps*. Otherwise, in the text, the abbreviation EPS has been used for Extracellular polymeric substances.

P. aeruginosa and their role in biofilm formation and function is given in the sections to follow (for Psl and Pel in chapter I, section 2, and for alginate in chapter II).

iv) Dispersal of biofilm

After maturation, the biofilm continues its life cycle by dispersing bacterial cells. After leaving the mature biofilm, these planktonic cells again attach to a new surface and start the development of another microcolony. Many studies have identified that the dispersal is initiated in response to various external environmental cues. Changes in availability of the following nutrients have been identified as signals that trigger dispersal: a) carbon sources, b) oxygen, c) nitric oxide (NO), and d) iron. In addition, many signalling molecules including the quorum sensing (QS)[2] signalling molecule AHL, diffusible fatty acids, peptides and D- amino acids have been found responsible for initiation of the process [reviewed in (109)] .

In *P. aeruginosa*, and other *Pseudomonas* species, active dispersal of the bacteria from biofilm is initiated by localized cell death in a subpopulation (79, 110). These dead cells are used as nutrients by other bacterial cells which provide these cells with sufficient energy to become 'dispersal cells' [reviewed in (109)]. These active cells leave the biofilm through 'local break out points' and swim away to start another lifecycle (111). In *P aeruginosa*, localized cell death is also thought to be a source of digestive enzymes which might play a role in the digestion of the exopolysaccharides and allow the release of bacterial cells [reviewed in (112)].

The intracellular level of c-di-GMP controls the mode of life in bacteria. Decrease in concentration of this secondary messenger leads to changes which result in switching from sessile to planktonic life again (113). Nitric Oxide has been identified as a signal in *P. aeruginosa* which activates the PDEs. Activated PDEs can lead to a decrease in c-di-GMP level in bacterial cell. Consequently the bacterial cells become motile and ready to disperse (113).

REGULATION OF BIOFILM FORMATION

Two important molecules involved in regulation are discussed below.

2 The term, quorum sensing is discussed in detail in subsequent section on page 15.

i) Cyclic diguanylate monophosphate

In the bacterial cell c-di-GMP is synthesized by DGCs from two molecules of GTP and degraded to 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or guanosine monophosphate (GMP) by PDEs (49). DGCs and PDEs are characterized by the presence of the conserved domain GGDEF (114) and EAL / HD-GYP (115)(116), respectively. Proteins containing GGDEF/EAL domains are mostly multi-domain proteins that contain some regulatory domains (117). These regulatory domains respond to environmental stimuli and consequently induce the DGC or PDE activity of the protein. In response to increased activity of DGCs and PDEs, localized concentration of c-di-GMP is changed. The effector proteins, containing specific c-di-GMP- binding domains, bind to c-di-GMP (118). The effectors become activated or inactivated in response to binding with c-di-GMP and regulate motility, attachment, exopolysaccharide biosynthesis, biofilm formation and dispersal of the biofilm [reviewed in (49)].

In *P. aeruginosa*, c-di-GMP plays its regulatory role in all steps of biofilm development, from attachment to dispersal. Flagellar and Type IV pili, important structures for initial attachment, are regulated by GGDEF/EAL-containing proteins such as MorA and FimX. MorA mutants showed an inability to form biofilm whereas FimX mutants lost polarity of pili expression (119, 120). As mentioned above (Page No. 8), SadC increases c-di-GMP, this results in the transition from reversible to irreversible attachment (50, 51). C-di-GMP controls the biosynthesis of exopolysaccharide at transcriptional and translational levels. FleQ represses the transcription of *pel* operon after binding to its promoter but, in the presence of c-di-GMP, FleQ is inactivated and the promoter is derepressed (121). Pel biosynthesis is controlled at post-transcriptional level by c-di-GMP-binding protein, PelD (122). C-di-GMP also regulates the exopolysaccharide alginate biosynthesis in *P. aeruginosa* (123). An important protein involved in alginate biosynthesis, Alg44, binds with c-di-GMP for its activity (124).

ii) Quorum sensing

After irreversible attachment, the bacterial cells residing close to each other start forming microcolonies. Bacterial cells within these microcolonies communicate with each other and develop coordination amongst the members of the community. This

signalling is transmitted through specific chemicals called autoinducers and this phenomena is called quorum sensing (QS) (125). QS is now widely recognized as an essential mechanism in many bacterial species. Most Gram negative bacteria regulate QS by production of autoinducer, *N*-acyl-L-homoserine lactones (AHLs) [reviewed in (126)]. This mechanism was first identified in a marine bacteria *Vibrio fischeri* which lives in symbiotic associations with many marine organisms and produces bioluminescence. The bioluminescence was seen only when the bacterial population-density was above certain threshold level. The bioluminescence was found to be associated with an increase in concentration of an autoinducer -(30-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL, 3) produced by *V. fischeri* cells [reviewed in (127)]. It is now known that *luxI/luxR* circuit controls this mechanism. In bacteria LuxI, an autoinducer, synthase, catalyzes synthesis of AHL. The receptor protein, LuxR, usually remains inactive and is continuously degraded in the cells. When AHL binds with LuxR, it becomes activated and activated LuxR binds to DNA and starts transcription of the target genes. This QS mechanism has been found essential for exopolysaccharide biosynthesis, virulence, biofilm formation, and biofilm related antibiotic resistance [reviewed in (128)].

In *P. aeruginosa* two sets of *luxI/luxR* type systems have been identified. The *lasI/lasR* regulated QS system produces and senses *N*-(3-oxododecanoyl)-L-homoserine lactone (129), and the *rhlI/ rhlR*-based system synthesizes and responds to *N*-butyryl homoserine lactone (130). In addition *P. aeruginosa* has a third QS system, PQS which synthesizes 2-heptyl-3-hydroxy-4-quinolone, and regulates both the *las* and *rhl* QS systems (131).

It has been shown that *P. aeruginosa* biofilm accumulates high concentrations of AHL molecules, i.e the concentration of *N*-(3-oxododecanoyl)-L-homoserine lactone recorded in biofilm was 630µM, whereas in effluent only 14 µM was found (132). This high concentration is enough to initiate QS based responses in biofilm (133). Many studies involving wild type and QS mutants have shown the effect of QS on biofilm development, architecture and exopolysaccharide biosynthesis (134-136). Interestingly, a recent study has shown a link between c-di-GMP, QS and exopolysaccharide biosynthesis in *P. aeruginosa*. The study showed that AHL signals activate the protein TpbA which dephosphorylates TpbB, a PDE, and consequently leads to decreased c-

di-GMP production (137). This reduction in intracellular concentration of c-di-GMP, negatively impacts on exopolysaccharide production and leads to reduced biofilm formation.

THE IMPORTANT EXOPOLYSACCHARIDES FOR *Pseudomonas aeruginosa* BIOFILM FORMATION

Three exopolysaccharides produced by *P. aeruginosa* have been identified as important for biofilm formation, architecture and function: Pel, Psl and alginate. Their detail is given below.

i) Molecular structure of Pel

The structure of Pel has not been resolved yet. A comparison of sugar composition of the matrix from *P. aeruginosa* strain PA14 and Pel-deficient mutant revealed that the matrix of the Pel-deficient mutant contained less glucose than the Pel-producing strain (107). The study indirectly suggested that Pel is made of glucose. In contrast, a recent study provided an analysis of the total polymeric substances produced by PA14 and showed that the most abundant extracellular carbohydrates present in a PA14 culture were O-antigen lipopolysaccharide and glycerol-phosphorylated cyclic β-(1,3)-glucans. They did not detect any other glucose rich exopolysaccharide (138).

ii) Pel biosynthesis

Pel synthesis and export machinery is encoded by *pel* operon which consist of seven genes: *pelA, pelB, pelC, pelD pelE, pelF* and *pelG* (107) and all these genes have been shown to be essential for Pel biosynthesis (139). These *pel* genes are conserved in most Gram-negative bacteria and a study showed that a mutation in a homologue of *pelG* in *Ralstonia solanacearum, ragG,* resulted in defective in adherence of the bacteria to a solid surface (139). A little experimental work has been done around the structure and function of the proteins encoded by the *pel* genes. Only two proteins, PelC and PelD, have been experimentally examined to date (122, 137, 140, 141). The function and structure of other proteins encoded by the *pel* operon have been suggested on the basis of sequence homology (139, 142, 143). Using these experimental evidences and

bioinformatics analysis of the proteins encoded by the *pel* operon, hypothetical models for Pel exopolysaccharide biosynthesis has been proposed by different researchers (140, 143).

Biosynthesis of polysaccharide starts with polymerization of sugar molecules which are available as activated nucleotide-sugars (144-146). These activated sugars are usually synthesized by the enzymes encoded by genes from the polysaccharide biosynthesis operon. Interestingly, the *pel* operon lacks any such genes suggesting that precursors for Pel biosynthesis are taken from the main pool of nucleotide-sugars (143). Recently it has been shown that AlgC, is involved in biosynthesis of glucose-1-phosphate, and *algC* negative mutants were not able to form Pel (147). It is possible that AlgC may be a common enzyme required for precursor synthesis for Pel, alginate and Psl. The nucleotide-sugars required for Pel biosynthesis are not known. On the basis of sugar composition analysis of exopolysaccharides produced by *P. aeruginosa* PAK strains, it is suggested that Pel is made up of glucose. This suggests that an activated-glucose may be used towards Pel biosynthesis. These activated sugars are transferred onto growing polymer by the enzymes called glycosyltransferases (GT). According to bioinformatics analysis, PelF is the only glycosyltransferase encoded by the *pel* operon (143). On the basis of sequence similarities it is predicted that PelF belongs to the GT-4 family (according to the classification of CAZy(carbohydrate-active enzymes database) and members of this family are retaining glycosyltransferases (148). The retaining glycosyltransferases transfer sugar residues onto the acceptor molecule by retaining the anomeric configuration of the sugar as it was in the donor substrate. No transmembrane (TM) helices are predicted in PelF which is suggestive of a cytosolic subcellular localization. It is predicted that a cytosolic PelF synthesizes Pel on the cytosolic face of the inner membrane. It is not known how this newly synthesized polymer is transported across the plasma membrane. The *pel* operon contains only one glycosyltransferase, PelF which suggests that, like alginate, Pel is a homopolymer. Therefore, it is likely that Pel translocation occurs via a Wzy-independent pathway. In addition, the *pel* operon lacks a gene for an undecaprenyl-phosphate glycosylphosphotransferase which is required in undecaprenyl-phosphate dependent translocation of polysaccharide from the cytosol to the periplasm [reviewed in (143)]. The newly polymerized Pel is likely to be translocated to the periplasm by PelD or PelE or PelG, because all of these proteins are

predicted to be located in the inner membrane and contain multiple TM domains [reviewed in (143)]. PelD binds with c-di-GMP and regulates the Pel biosynthesis (122). The role of c-di-GMP can be linked with Pel production because when the intracellular concentration of c-di-GMP was manipulated by deletion of tyrosine phosphatase, TpbA, the amount of Pel production and pellicle formation was also changed (137). PelG is predicted to be an inner membrane protein which contains 12 TM domains (149). On the basis of structural homology, PelG has been shown to be similar to NorM from *V. cholerae* (PDB ID:3MKU). NorM belongs to the multidrug and toxic compound extrusion (MATE) family of proteins (150). Members of this family of proteins are involved in extrusion of molecules across the inner membrane. PelG could be involved in export of Pel polysaccharide from the cytoplasmic face of the inner membrane to the periplasm. PelE, the third inner membrane protein, is predicted to have two transmembrane domains which orient the protein in such a way that the C-terminus becomes localized to the periplasm. The C-terminus of the protein is predicted to contain 4-6 copies of tetratricopeptide-like repeats (TPR), the protein–protein interaction motif. Previously it has been shown TPR motifs are involved in the assembly of large protein complexes (151, 152), therefore, PelE, may be involved in interaction with other TPR-containing proteins in the periplasm. The homology modelling suggests that the N-terminus of PelB forms a domain which has similarity with TPR-containing anaphase-promoting complex/cyclosome subunit Cdc 16/Cut9 [PDB ID: 2XPI; (153)] and is predicted to contain TPR motifs. It can be proposed that the C-terminus of PelE and N-terminus of PelB are involved in protein interactions to make a scaffold. PelC has been proposed as a lipoprotein in which Cystein-19 is lipidated and contains a C-terminal amphipathic α-helix which is inserted in the outer membrane (OM) in a Wza-like manner. This C-terminus domain has been shown to be essential for Pel biosynthesis. PelA is a large 105 kDa protein in which an N-terminal domain is structurally similar to glycoside hydrolases (PDB ID:2AAM) and a C-terminal domain shows similarity to carbohydrate esterase [PDB ID:2VYO; (154)]. The glycosidic hydrolase activity of PelA suggests that PelA may play a role similar to AlgL. AlgL hydrolyses alginate which escapes into the periplasm (155). Therefore, it is possible that any Pel that escapes into the periplasm is hydrolyzed by PelA.

Pel biosynthesis can be summarized as PelF polymerizing a homopolymer of glucose which is transferred from the cytoplasm to the periplasm by PelE /PelD/ PelG.

iii) Molecular structure of Psl

Previous studies suggests that Psl is composed of galactose, D-mannose, D-glucose, and L-rhamnose (106, 156) Recently the molecular structure of Psl is revealed (157). The study showed that Psl exopolysaccharide is composed of pentasaccharides repeating units and each unit is composed of three D-mannose, one D-glucose, and one L-rhamnose.

iv) Psl biosynthesis

Biosynthesis of Psl in *P. aeruginosa* is under the control of the *psl* operon which contains 15 co-transcribed genes: *pslA-pslO* (158). Interestingly, only 11 of the 15 gene products of the *psl* operon were found to be essential for Psl biosynthesis. The product of *pslB* has redundant function with WbpW, an enzyme which has similar activity to that predicted for PslB (157). Deletion of the last three genes of the operon, *pslMNO*, has shown no effect on the ability of *P. aeruginosa* to produce Psl (157).

Although the Psl biosynthesis mechanism has not been established experimentally, the repeating unit structure of Psl and bioinformatics analysis of the proteins encoded by the *psl* operon suggest that Psl biosynthesis bears a resemblance to the undecaprenyl diphosphate-based biosynthesis of *E. coli* group-1 capsular and extracellular polysaccharides. Therefore the Psl biosynthesis pathway resembles a Wzy-dependent pathway. This hypothesis is further strengthened by the finding that three proteins encoded by the corresponding genes of the *psl* operon PslA, PslD and PslE show sequence similarity with WbaP, Wza, and Wzc, respectively(143). The role of gene products of *psl* operon is presented in a recent review (143).

A detailed account of alginate is discussed in chapter II.

ROLE OF EXOPOLYSACCHARIDES IN BIOFILM

i) Role of Pel in biofilm

Pel is synthesized in most *P. aeruginosa* strains but strains with intact *psl* loci depend more on Psl contributing to biofilm formation and architecture. However, *P aeruginosa* PA14 relies mainly on the Pel exopolysaccharide because it lacks Psl (107). This strain produces biofilm at the air-liquid interface (A-L) and it was observed that a *pel* transposon insertion mutant failed to produce A-L biofilm (107, 158). This finding led to further detailed investigation of the role of Pel in biofilm formation. Pel has been shown to be required for adherence to abiotic surfaces especially at the later stages of the biofilm in PA14 (159). Recent studies have suggested a redundant role for Pel in *P aeruginosa* PAO1 biofilm, especially in the presence of Psl. However, in the absence of Psl, Pel has a clearer role in biofilm formation (160). In contrast, a recent study has demonstrated a distinct role for Pel in biofilm formation and suggested that, in addition to Psl, Pel was required for structural stability of mature biofilms (161).

Pel has also been identified as an extracellular matrix component which mitigates the effects of antibiotics and may play a vital role in protection of the bacteria (162). Studies demonstrated that Pel-producing PA14 strains showed increased resistance to aminoglycoside antibiotics when compared to Pel-deficient strains (162). In contrast, other authors have reported contradicting results which showed that Pel-deficient mutants were more resistant to the aminoglycoside, tobramycin when compared to wild type PA14 strains (163). The two studies used different methods and conditions to assess antibiotic resistance which might have affected the results of their experiments. Colvin *et al.* (162) grew biofilms on polycarbonate filters for 48 hours and then the filter was transferred to solid medium containing respective antibiotics and incubated for 24 hours. Viable bacterial cells were counted to see the effect of antibiotics. It was found that absence of Pel renders PA14 cells more susceptible to aminoglycosides when grown as biofilm. Whereas, Khan *et al*. (163) grew cells in 96-well microtiter plates in liquid medium for 24 hours. These one day old biofilms were washed and treated with respective antibiotics for 24 hours. It was found that in absence of Pel PA14 cells were more resistant to aminoglycosides. This difference of media conditions and duration of growth might have affected the exopolysaccharide production and distribution in biofilm formation. Owing to the significance of Pel in

antibiotic resistance and architecture of the biofilm, it is imperative to further investigate its regulation, biosynthesis and role in *P. aeruginosa* biofilm.

ii) Role of Psl in biofilm

Psl is found in two forms: a larger form associated with cells and a smaller soluble form. It is not yet clear how the smaller form of Psl is produced. Possibly, it may be produced by cleavage or breakdown of the longer cell-associated Psl. Alternatively, there could be another unrecognized mechanism to generate this form of Psl. The long cell-associated form of Psl forms blebs of Psl on the cell surface which can be observed by scanning electron microscopy (SEM) (164). Psl has been shown to be important for initial attachment of non-motile cells to both biotic and abiotic surfaces (157, 164, 165). Psl aids structural stability of the biofilm and is required for the typical architecture of the biofilm (156). Psl also plays an important role in protection of bacterial cells from innate immune effectors, complement and neutrophils (166). Recently it has been shown that Psl also stimulates the production of c-di-GMP in surrounding bacterial cells suggesting a regulatory role of Psl in biofilm formation and maintenance (167).

iii) Role of alginate in biofilm

Most of the early studies investigating *P. aeruginosa* biofilms were conducted using mucoid (alginate overproducing) strains (168-170). Mucoid strains isolated from chronically infected lungs of cystic fibrosis patients were able to produce copious amount of alginate when grown on agar plates in the laboratory. Mucoid colonies, encased in matrix made of alginate, appear more slimy when compared to non-mucoid strains (171). Alginate has been shown to be a very important matrix component for retention of water due to its gel-forming ability and protects the bacterial cells from the harmful effects of desiccation. Although direct evidence of the amount of water retained by alginate is not known, a similar exopolysaccharide, hyaluronic acid, can bind up to 1 kg of water per gram of the polysaccharide (172, 173). The degree of acetylation of alginate also plays an important role in its ability to retain water. A high degree of O-acetylation of the residues present in alginate increase its solubility by reducing the chance of interaction between alginate and cations. Alginates without O-acetyl residues show increased binding of divalent cations and decreased polysaccharide solubility

(172, 173). Alginate protects bacterial cells from the host immune response and antimicrobial drugs (174-176). Most of the host immune cells such as neutrophils and activated macrophages kill bacterial cells by releasing free radicals. Alginate can protect bacterial cells by scavenging the free radicals (174, 175).

iv) Role of exopolysaccharides in the architecture of the biofilm

The production and quantity of exopolysaccharides influences the architecture of biofilm. Studies show that mutants lacking exopolysaccharides produce biofilms which are structurally different from those produced by the wild type strains. For example, *V. cholerae* deficient in exopolysaccharides (177) and *E. coli* lacking colanic acid (102) failed to form 3D biofilm architecture. The fruiting body-like biofilms formed by wild type *B. subtilis* were absent in the mutants lacking exopolysaccharides and the secreted protein TasA (90). Less is known about the effects of polysaccharides on the architecture of *P. aeruginosa* biofilms. Some studies have been carried out to investigate the role of alginate in biofilm architecture (108, 178, 179). Although it has been suggested that alginate is not necessary for *P. aeruginosa* biofilm formation (179), however, biofilm architecture is influenced in the presence of alginate (27). Some recent studies have shown the impact of Psl in biofilm architecture (11, 165, 180). Further investigations are required to decipher the role of Pel in the architecture of *P. aeruginosa* biofilms.

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CHAPTER II

Bacterial biosynthesis of alginates

Iain D. Hay, Zahid Ur Rehman, Aamir Ghafoor, Bernd H. A. Rehm

Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

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ABSTRACT

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

INTRODUCTION

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

Two bacterial genera are also capable of producing alginate as an exopolysaccharide, *Pseudomonas* and *Azotobacter*. They produce alginates with marked differences in material properties and function. One of the model organisms for alginate production is the opportunistic pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is of great importance to cystic fibrosis (CF) patients where it is the leading cause of morbidity and mortality. In *P. aeruginosa* alginate has been shown to be important for the formation of thick highly structured biofilms (3, 4). The CF lung provides a unique environment to the pathogen which induces the bacteria to overproduce alginate, producing a thick biofilm, protecting itself from the host immune response and antibiotic treatment, while contributing to the clogging of the lung (5-9). In *Azotobacter*, the alginate produced can have a higher concentration of G-blocks and thus is relatively stiff, this alginate is used in the formation of desiccation resistant cysts (10).

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

derived alginates underperform. Bacterial alginates may provide a base for the production of alginate with more defined chemical and material properties, furthermore, alginates could potentially be tailor made to have certain properties. Already, epimerases from *Azotobacter vinelandii*, which convert M residues to G residues, have been used to modify the G content and thus material properties of seaweed derived alginates (1), and alginates from mutant *Pseudomonas fluorescence* can be produced that lack any G residues (polymanuronan) (11, 12).

GENETICS OF BACTERIAL ALGINATE BIOSYNTHESIS

The work of Darzins and Chakrabarty (13) first demonstrated many of genes involved in alginate production in *P. aeruginosa*, using complementation studies. To date at least 24 genes have been found to be directly involved in alginate production in *P. aeruginosa* (14) (Table 1) With the exception of *algC* all the structural genes involved in alginate biosynthesis are clustered in a single operon, first described by Chitnis and Ohman (15). The cluster consists of 12 genes:*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, and *algA* located at approximately 3.96 Mb on the PAO1 genome map. This operon is under the tight control of a promoter located upstream of *algD* (16-18). Within this operon are the genes encoding proteins involved in alginate precursor synthesis (*algD* and *algA*) ; proteins that modify the nascent alginate chain (*algI*, *algJ*, and *algF* for acetylation (19), *algG* for epimerization (20), and *algL* for degradation (21)); the putative outer membrane porin (*algE*) (22, 23). The products of the *alg8* gene is thought to be involved in the transfer of GDP-mannuronic acid across the cytoplasmic membrane and have recently been shown to play a role in the polymerisation process (24). The product of the *alg44* gene is thought to be involved in the post-translational regulation of alginate (25, 26). The functions of the products of the remaining two genes in the operon, *algK* and *algX*, are unclear but there products are essential for production of alginate and are thought to play some sort of structural or protective role, guiding the alginate polymer through the periplasmic space (27, 28). Some of the genes involved in alginate production encode proteins that are not exclusively involved in alginate biosynthesis. This is true for the *algC* gene, encoding a phosphomannomutase which is involved in precursor synthesis. This gene product is also involved in rhamnolipid and lipopolysaccharide biosynthesis (15, 29, 30) and expression is driven from its own

promoter (31). Also several of the regulatory proteins do not act exclusively on alginate biosynthesis genes, as will be discussed below. The multiple roles of these proteins would suggest that alginate production is part of a much larger, complex metabolic and regulatory network.

Table 1 Genes involved in alginate biosynthesis and their function (or putative function). Adapted from Rehm (2002) (14)

Gene	Gene product	Reference
algD	GDP mannose dehydrogenase	(32)
alg8	Glycosyltransferase/polymerase function?	(33)
alg44	c-di-GMP binding regulation/ membrane fusion?	(25)
algK	Periplasmic scaffold?	(27)
algE (algJ)	Outer membrane alginate porin?	(22, 34)
algG	Mannuronan C-5-epimerase	(20)
algE1-E7	*Azotobacter* extracellular epimerases	(35)
algX	Periplasmic unknown function/scaffold/sequesters MucD	(28)
algL	Alginate lyase	(36)
algI	O-Acetylation	(19)
algJ (algV)	O-Acetylation	(19)
algF	O-Acetylation	(19)
algA	Phosphomannose isomerase/GDP mannose pyrophosphorylase	(37)
algB	Member of ntrC subclass of two-component regulators	(38)
algC	Phosphomannomutase	(29)
algH	Unknown	(39)
algR	Regulatory component of two-component sensory transduction system	(38)
algQ	Histone like transcription regulator. AKA *algR2*	(40)
algP	Histone-like transcription regulator. AKA *algR3*	(41)
algZ	AlgR cognate sensor. AKA fimS (PA5262)	(42)
amrZ	Arc-like DNA binding protein. Formally called *algZ* (PA3385)	
algU	Homologue of *E. coli* σ^E global stress response factor/σ^{22}	(43)
mucA	Anti σ factor	(43)
mucB	Anti σ factor	(44)
mucC	Regulator?	(45)
mucD	Homologue of *E. coli* serine protease DegP	(46)
algW	Homologue of *E. coli* serine protease DegS	(44)
mucP	Homologue of *E. coli* RseP protease involved in AlgU RIP cascade	(47)
mucE	Periplasmic or outer membrane protein involved in AlgU RIP cascade	(47)
mucR	Alginate specific diguanylate cyclase (c-di-GMP synthesizing)	(26)

BIOSYNTHESIS OF ALGINATE

The first bacterial alginate biosynthesis pathway was proposed in 1975 by Pindar and Bucke in *A. vinelandii* (48). A combination of complementation studies and overexpression studies has provided a convincing model for the biosynthesis of alginate in *P. aeruginosa*. This can be broken down into four stages: (i) precursor synthesis, (ii) polymerisation and cytoplasmic membrane transfer, (iii) periplasmic transfer and modification, and (iv) export through the outer membrane (49) (Figure. 1).

By far the best understood part of alginate biosynthesis in *P. aeruginosa* is the synthesis of the precursor GDP-mannuronic acid in the cytosol. Radio-labelling studies have shown that the synthesis starts with the entry of 6 carbon substrates to the Entner-Douderoff pathway (KDPG pathway), resulting in pyruvate, which is channelled towards the tricarboxylic acid (TCA) cycle, while oxaloacetate from the TCA cycle can be converted to fructose-6-phosphate via gluconeogenesis (50, 51). The conversion of fructose-6-phosphate to mannose-6-phosphate is catalysed by the phosphomannose isomerase (PMI) activity of the bifunctional protein AlgA (PMI-GMP) (52). AlgC (phosphomannomutase) directly converts mannose-6-phosphate to mannose-1-phosphate (53). The GDP-mannose pyrophosphorylase (GMP) activity of AlgA (PMI-GMP) catalyses the conversion of the activated mannose-1-phosphate to GDP-mannose with the hydrolysis of GTP (37). The GMP activity of this enzyme favours the reverse reaction, but the constant conversion of GDP-mannose to GDP-mannuronic acid by the activity of AlgD (GDP-mannose-dehydrogenase) shifts the reaction towards GDP-mannuronic acid and alginate production. This AlgD catalysed reaction is essentially irreversible and provides the direct precursor for polymerisation, GDP-mannuronic acid (54). This and the high intracellular levels of GDP-mannose would indicate that this AlgD catalysed step is a limiting step and/or important kinetic control point in alginate biosynthesis (32).

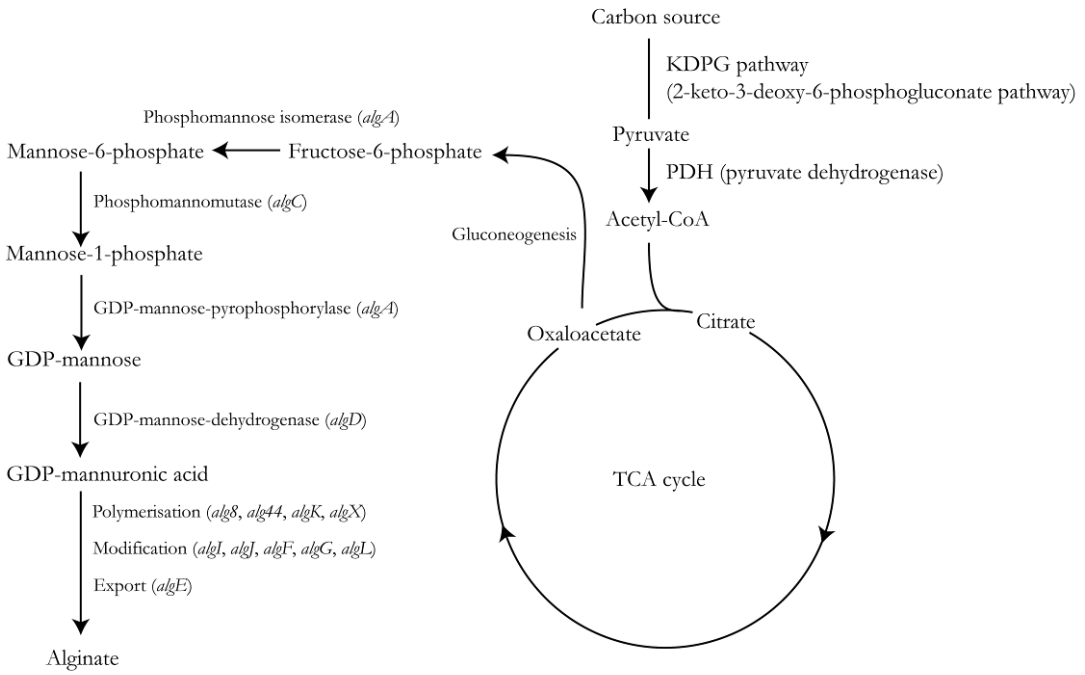

Figure 1. Proposed alginate biosynthesis pathway in *P. aeruginosa* (adapted from Remminghorst & Rehm, 2006 (49).

Polymerisation and translocation are the poorest understood processes in alginate biosynthesis. This is mainly due to the fact that at present no polymerase or polymerase complex has been purified, and up until recently no alginate synthesis activity had been demonstrated in vitro (24). Alg8 is thought to be a good candidate for the alginate polymerase enzyme. It shares homologies with class II glycosyltransferases, which catalyse the transfer of sugar residues from an activated donor to an acceptor molecule, which can be a growing carbohydrate chain (24, 55). Furthermore, structural similarities were observed when compared to functionally related enzymes such as AcsAB (cellulose synthase) (56) and Chs1 (chitin synthase) (49). Overproduction of Alg8 results in a 15-fold increase in the levels of alginate biosynthesis, this suggests that Alg8 is a key bottleneck and further suggests that it is the catalytic subunit of the multiprotein complex. AlgG, AlgK and AlgX are thought to provide some kind of periplasmic scaffold, along with the outer membrane protein AlgE, to guide and protect the nascent alginate chain from lyase degradation (11, 57-60). Deletion mutants of these proteins did not show alginate production but showed secretion of free uronic acids, indicating that polymanuronate is being formed and subsequently degraded by the periplasmic alginate lyase (AlgL). This suggests that they are not playing a direct role in

the polymerisation process but may be playing more of a structural/protection from alginate lyase degradation (discussed below) role (11, 36, 59).

It has been suggested that Alg8 and Alg44 interact and play an important role in the polymerase complex (61). Recently, it was shown in complementation studies that Alg44 is required for alginate polymerisation. Homology based secondary structure predictions showed some level of similarities of the C terminal half of Alg44 to the membrane bridging protein MexA. This suggests a function of Alg44 as part of the periplasmic scaffold, where it may provide a bridge between the cytoplasmic membrane protein Alg8 and the outer membrane export protein AlgE (25) (Figure. 2). Additionally the cytosolic N terminal half of Alg44 has been shown to possess a functional and essential PilZ domain (25, 62). PilZ domains are involved in binding of the regulatory molecule bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (63). This may suggest an additional regulatory role for Alg44, as shown previously for the cellulose synthase.

Modification of alginate in bacteria occurs almost exclusively in the periplasm, which suggested that alginate is synthesised as polymannuronate and that modification occurs at polymer level. Three classes of alginate modifying enzymes have been described: transacetylases (19, 64-66), mannuronan C5-epimerases, (11, 12, 20) and lyases (67, 68). In *P. aeruginosa* AlgI, AlgJ (called AlgV in *Azotobacter*), and AlgF were shown to form the acetylation complex (19, 64-66). Transacetylation occurs at the O-2 and/or O-3 position and only occurs at mannuronic acid residues. Acetylation of these residues prevents their epimerization to guluronic acid residues by AlgG. It also prevents degradation of the alginate chain by AlgL (20, 66, 69). Thus, the acetylation of alginate indirectly controls epimerisation and length of the alginate polymer.

Mannuronan C-5-epimerase introduces guluronic acid residues in to the polymannuronate. There has only been one such enzyme identified in *P. aeruginosa*, AlgG, whereas *A. vinelandii* has a total of eight epimerases: the periplasmic AlgG and the extracellular AlgE1-AlgE7 (35, 70). These Ca^{2+} dependent extracellular epimerases have differing specificities for M G composition and introduce different distributions of guluronic acid residues into their substrate molecules, additionally AlgE7 has alginate lyase activity. This might explain the highly variable alginate produced by *A. vinelandii*, which is important for cyst formation and differentiation (35, 71, 72).

AlgL, catalyses the β-elimination reaction leading to the degradation of alginate. The exact biological function of alginate lyase has not been fully understood but alginate producing bacteria are not able to reutilize the alginate as a carbon source. There is conflicting evidence as to the necessity of AlgL in the biosynthesis of alginate: Albrecht and Schiller (68) demonstrated that loss of the *algL* gene resulted in a complete loss of alginate biosynthesis which could be restored by complementation with the *algL* ORF in trans but not with an ORF encoding AlgL variants lacking lyase activity (containing mutations in the putative active regions); whereas Jain and Ohman(36) showed that loss of *algL* does not result in loss of alginate biosynthesis but leads to accumulation of alginate in the periplasm and eventually the lysis of the cells. Additionally AlgL has been shown to be dispensable for both alginate biosynthesis and growth in *Azotobacter vinelandii* (73). It has been suggested that AlgL may function in controlling the length and molecular weight of the alginate polymer (74), providing short alginate polymers to "prime" the polymerisation of alginate (74), or to clear the periplasm of misguided alginate (36, 67). It should be noted that in addition to AlgL and its orthologues found in all alginate producing bacterial species, additional alginate lyase proteins have been identified in both alginate producing and non-producing bacteria (for review see Wong, T.Y et al (69)). These enzymes have differing residue specificities and cellular localisations. Among *Pseudomonas* species there has been one other protein identified to have alginate lyase activity, PA1167 (75). Among *Azotobacter* species at least four other lyases have been identified: the epimerase AlgE7, and three others, AlyA1, AlyA2 and AlyA3 (76).

AlgE (called AlgJ in *Azotobacter*) is the putative porin through which the alginate is secreted. Electrochemical analysis of AlgE showed that it functions as an anion selective pore (22). This protein has been shown to be essential for the secretion of intact alginate and can be detected in the outer membrane of mucoid, alginate overproducing, strains of *P. aeruginosa* but is absent in non-mucoid strains (60, 77).

REGULATION OF BACTERIAL ALGINATE BIOSYNTHESIS

The regulation of alginate biosynthesis is complex and involves transcriptional and post translational regulation, as well as several hypermutable regions of the genome which

can switch on or off alginate production. Both alginate biosynthesis specific regulators and globally acting regulators influence alginate biosynthesis. Transcriptional regulation of alginate biosynthesis in *P. aeruginosa* can be loosely divided into two different types: environmental stimuli based regulation; and a "genotypic switch" based form of regulation.

The "switch" loci contains the genes *algU*, *mucA*, *mucB*, *mucC* and *mucD*. *algU* encodes an extra-cytoplasmic alternate σ^{22} factor, which is at the apex of a hierarchy of regulators involved in alginate biosynthesis and is ultimately required for transcription starting from the *algD* promoter and thus expression of the core alginate genes (78-81). This region shows high similarity to the well characterised σ^{E} region in *E. coli*, containing the genes *rpoE* (encoding the σ^{E}), *rseA*, *rseB*, *rseC*. This operonic structure appears to be conserved among most of the gamma proteobacteria (82). In *E. coli* the σ^{E} signal transduction pathway is part of an envelope stress response system, whereby external stresses such as temperature fluctuations, are thought to lead to misfolding of outer membrane proteins in the periplasm (83). This leads to activation of the sigma factor by a regulated intramembrane proteolysis (RIP) cascade(84). It is thought it functions in a similar way in *Pseudomonas*. The key proteins of the *Pseudomonas* alg/muc RIP cascade were elucidated over a decade ago. These are the sigma factor AlgU (σ^{22}) and the intramembrane anti-sigma factor MucA. AlgU is required for transcription of the alginate operon as well as transcription of several of the regulatory operons (including its own). MucA inhibits the transcriptional activation activity of AlgU by sequestering it at the cytosolic side of the inner membrane preventing it from binding to RNA polymerase (16, 85-87). The periplasmic protein MucB has been shown bind to the periplasmic side of MucA and play a negative regulatory role in alginate biosynthesis by protecting MucA from proteolysis and aiding in the sequestering of AlgU (44, 88). It has been shown that mutation of both MucA and/or MucB leads to transcriptional activation of the *algD* promoter and a highly mucoid (alginate overproducing) phenotype (87, 89), indicating that the cytosolic AlgU sequestering activity of MucA is dependent on the periplasmic MucB protein. Several of the proteases involved in the RIP cascade degradation of MucA have recently been identified. Wood and Ohman (44, 88) demonstrated that in responce to envelope stress MucA is initially cleaved by the periplasmic protease AlgW (*E. coli* DegS homologue)

and subsequently cleaved on the cytosolic side by the intramembrane protease YaeL (*E. coli* RseP/YaeL homologue) leading to the release of AlgU. The PDZ activating domain (de-repression) of AlgW is activated by the the C-terminus of particular misfolded proteins, in particular a periplasmic or outer membrane located protein called MucE (47). A third periplasmic protease, MucD, appears to be playing a role antagonistic to that of AlgW. Deletion mutations of MucD lead to a mucoid phenotype indicating a negative regulatory role (46). MucD appears to be involved in the degradation of misfolded proteins in the periplasm that would outherwise activate (de-repress) AlgW (47, 88) (Figure. 2). The role of MucC remains unclear. It should be noted that the alternate sigma factor, AlgU, released in this RIP cascade does not exclusively activate transcription of the alginate operon but is involved in the the transcription of other genes with diverse functions (79, 90).

There appears to be an addition level of "control" over this cascade: One study found that over 80% of mucoid *P. aeruginosa* isolates obtained from CF patients contained mutations in the *mucA* gene (91). Most of these mutations result in a premature stop codon and a truncated MucA. The most common mutation found in mucA was the deletion of a single guanine in a homopolymeric stretch of five G residues, resulting in a frameshift and subsequent recognition of a premature stop codon (*mucA22* allele). Removal of MucA, the anti-sigma factor at the base of this complex RIP cascade means there is nothing to sequester the sigma factor AlgU and thus transcription from the *algD* promoter can proceed.

In addition to AlgU, several other proteins are required to initiate transcription of the alginate operon. AlgR is a response regulator part of a two component regulator that binds to three sites in the *algD* promoter, the sensory component of this regulatory pair is AlgZ (FimS) and strangely is not required for transcription of the alginate operon (38). AlgB is also part of a two component regulator and binds to one site on the *algD* promoter, again its activity is apparently independent of its sensor kinase KinB (38). AmrZ (originally called AlgZ), an Arc-like DNA binding protein, binds to one site on the *algD* promoter (42, 92). These proteins are all also involved in the regulation of other diverse processes.

Recently a level of post translational regulation of alginate biosynthesis has emerged. The alginate biosynthesis protein Alg44 contains a c-di-GMP binding/sensing

PilZ domain in its C-terminus, indicating that c-di-GMP may be playing a regulatory role in alginate biosynthesis (25, 62). C-di-GMP is an important bacterial secondary messenger that has been linked to the post-transcriptional regulation of diverse processes such as motility, exopolysacharide production and virulence. Recently it has been demonstrated that one particular c-di-GMP synthesizing protein, MucR, specifically influences the levels of alginate biosynthesis in *P. aeruginosa* (26) (Figure. 2).

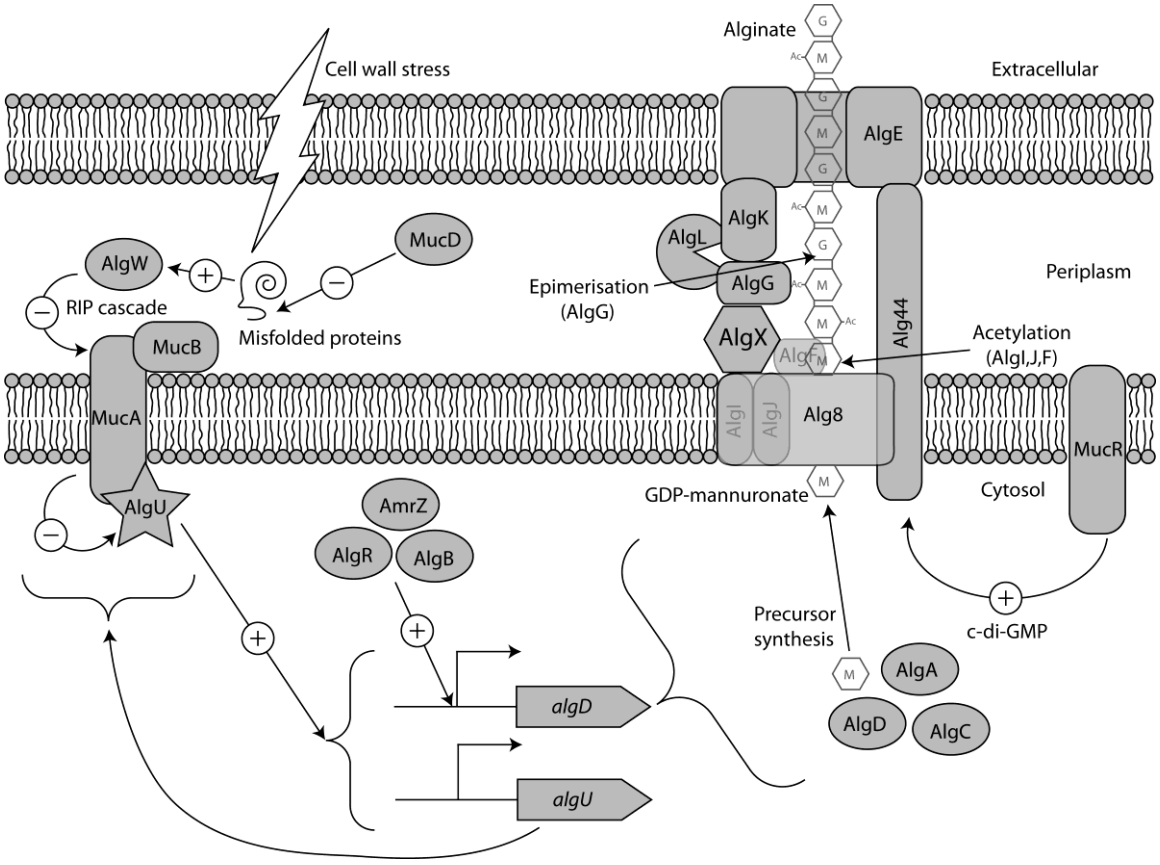

Figure 2. Overview of alginate biosynthesis in *P. aeruginosa*.

APPLICATIONS OF BACTERIAL ALGINATES

Since its discovery alginate has been used for many commercial purposes. Due to its water holding, gelling, viscosifying and stabilising properties, alginate of algal origin is used extensively in the food industry as a food additive. Based on its high biocompatibility (93), abundance and availability of the source, and relatively low cost to produce, alginates are increasingly being used in the medical field (94). Alginate

dressings are widely used to treat exuding wounds. These dressings are generally comprised of fibres of stiff calcium alginate, which soften and can "mould" to the area applied. In contrast to traditional wound dressings which adhere to a wound and can be painful and difficult to remove and re-apply, these dressings are advantageous as they do not adhere to the wound and can be removed with a saline wash (94). Alginate has also been used for the controlled release of drugs; the drugs are entrapped in alginate beads, from which the drug can by slowly released (95). A similar method has been applied to encapsulate functional cells to be transplanted in to a subject (iso-, allo- or xenotransplantation) making the cells much less immunoreactive and thus less likely to be rejected by the subject, for example parathyroid tissue to treat hypoparathyroidism (10, 96-99). Also, recently alginate encapsulation has been used successfully as a method to orally deliver a DNA based vaccines (100). To date the vast majority of the alginate used for commercial and medical purposes is obtained via the harvesting of brown seaweeds. These naturally occurring alginates are poor with respect to their purity and consistency of polymer composition. They are often contaminated with protein and other immunogenic compounds which require extensive downstream processing to remove. The relative high price of the commercial production of bacterial alginates cannot compete with the low price of seaweed derived alginates. The potential for bacteria to produce high quality alginates with defined material properties for use in high value applications, such as those in the medical field, may provide a niche for the commercial production of bacterial alginates. Many of the material properties of alginate depend on the monomer composition, the degree of acetylation, or the length of the polymer as well as the degree and type of modifications. A knowledge of the mechanisms of the bacterial alginate modifying enzymes combined with the potential to manipulate and exploit these enzymes by genetic and protein engineering may allow for the production of "tailor made" bacterial alginates with "user defined" material properties (18, 61). These alginates could be directly produced from the bacteria or produced from the cheaper seaweed source or as polymannuron from *P. fluorescens* (11, 12) and later modified with bacterial enzymes. Indeed this has already been demonstrated with the use of the extracellular epimerases secreted by *Azotobacter vinelandii* to treat polymannuron to produce alginates not found in nature with useful material properties (1, 101).

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AIMS OF THE STUDY

Owing to the clinical importance of bacterial biofilms, it has become imperative to understand the process of biofilm formation. The ubiquity, versatility and ability to survive in diverse ecological niches are the attributes of *P. aeruginosa* as well as its clinical relevance which have made it a model organism for biofilm study. The exopolysaccharides required for the architecture and function of the *P. aeruginosa* biofilm have become a main focus of research. A better understanding of these macromolecules and their role in biofilm will help us to make more informed decisions in designing better treatment and control strategies.

- This study aimed to explore the role of Psl, Pel and alginate in biofilm formation. The main focus was to understand that how the absence of one or two exopolysaccharides influences the architecture of the biofilm.
- The study also intended to investigate temporal and spatial regulation of these exopolysaccharides in mucoid and non mucoid biofilms.
- These days, extensive research is being undertaken to explore the structure and biosynthesis of the exopolysaccharides in *P. aeruginosa*, specifically, alginate and Psl. However, less is known about the exopolysaccharide, Pel. This study was designed to initiate the exploration of Pel biosynthesis by investigating the only glycosyltransferase encoded by the Pel biosynthesis operon.

CHAPTER III

The role of various exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture: Generation of isogenic exopolysaccharide biosynthesis knock out mutants

Aamir Ghafoor, Iain D. Hay, Bernd H.A. Rehm

Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic human pathogen and has been established as a model organism to study bacterial biofilm formation. At least three exopolysaccharides (alginate, Psl and Pel) contribute to the formation of biofilms in this organism. Here mutants deficient in the production of one or more of these polysaccharides were generated to investigate how these polymers interactively contribute to biofilm formation. Confocal laser scanning microscopy of biofilms formed in flow chambers showed that mutants deficient in alginate biosynthesis developed biofilms with a decreased proportion of viable cells when compared to alginate-producing strains, indicating a role of alginate in viability of cells in biofilms. Alginate-deficient mutants showed enhanced extracellular DNA (eDNA)-containing surface structures impacting on biofilm architecture. PAO1 Δ*pslA* Δ*alg8* overproduced Pel, and eDNA showing meshwork-like structures presumably based on an interaction between both polymers were observed. The formation of characteristic mushroom-like structures required both Psl and alginate, whereas Pel appeared to play a role in biofilm cell density and/or the compactness of the biofilm. Mutants producing only alginate, i.e., deficient in both Psl and Pel production, lost their ability to form biofilms. A lack of Psl enhanced the production of Pel, and absence of Pel enhanced production of alginate. The function of Psl in attachment was independent of alginate and Pel. A 30% decrease in Psl promoter activity in the alginate-overproducing MucA-negative mutant PDO300 suggested inverse regulation of both biosynthesis operons. Overall, this study demonstrated that the various exopolysaccharides and eDNA interactively contribute to the biofilm architecture of *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa has been widely studied as a model organism for biofilm formation. *P. aeruginosa* is an opportunistic human pathogen which causes infection in burn wounds and in the lungs of patients suffering from the genetic disease called cystic fibrosis (CF). The ability to form biofilms is critical for its survival in the CF lung environment and enhances its resistance to antimicrobial treatment and host defense mechanisms (1). *P. aeruginosa* biofilms are also common on medical devices, such as contact lenses and catheters (2). At least three exopolysaccharides have been shown to be produced by *P. aeruginosa*: alginate, Psl and Pel (3). Each of these exopolysaccharides has been found to be involved in biofilm formation (3).

Alginates, which are overproduced by *P. aeruginosa* after infection of CF patients, are linear polyanionic exopolysaccharides composed of uronic acids (4). Alginate-overproducing mutants form large finger-like microcolonies compared to wild-type strains (5). Alginate has been shown to contribute to decreased susceptibility of biofilms to antibiotic treatment and human antibacterial defence mechanisms (6, 7).

The Psl polysaccharide is rich in mannose and galactose and is involved in initial attachment and mature biofilm formation (8). Psl is produced during planktonic growth, mediating attachment to surfaces and contributing to microcolony formation. In mature biofilms, Psl is associated with the caps of mushroom-like microcolonies, forming a peripheral meshwork covering the cap region (8, 9).

Pel is a glucose-rich, cellulose-like polymer essential for the formation of a pellicle at the air-liquid interface (10). Increased Pel production has also been associated with the wrinkled colony phenotype (11). It has recently been shown that Pel plays a role in cell-to-cell interactions in *P. aeruginosa* PA14 biofilms, providing a structural scaffold for the community at early stages of biofilm formation (11).

Besides the important role of the exopolysaccharides in biofilm formation, extracellular DNA (eDNA) has been shown to be an important component of the biofilm matrix (12, 13). eDNA was found to mediate cell-cell interactions in biofilms (13). Moreover, it was shown that removal of eDNA by DNase treatment at initial stages of biofilm formation interfered with maturation of the biofilm. However, in mature biofilms such DNase treatment showed little impact on biofilm architecture (14). eDNA was found to be present mostly in the stalk region of microcolonies (13). A

recent study has suggested that Psl and eDNA are spatially separated from each other, with Psl being present at the periphery of the biofilm and eDNA mostly in the Psl-free biofilm matrix (8).

This study aims to shed light on how these exopolysaccharides and eDNA contribute to biofilm formation and architecture, particularly in view of synergistic effects between the different exopolysaccharides.

MATERIALS AND METHODS

i) Bacterial strains and growth conditions

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S1 in the supplementary material. Luria-Bertani (LB) medium, purchased from Neogen (MI, USA), was used to grow all *Escherichia coli* strains at 37°C. When needed, the following antibiotics were added to the media at the indicated concentrations: ampicillin, 75 μg/mL, and gentamicin, 10 μg/mL. *P. aeruginosa* PAO1 and PDO300 (15) and their isogenic mutants were grown in LB medium, Pseudomonas isolation (PI) broth (20 g of peptone, 10 g of K_2SO_4, 1.4 g $MgCl_2$. $6H_2O$, 25 mg of triclosan, and 20 mL of glycerol per liter) or Pseudomonas isolation agar (PIA) medium at 37°C; and if required, gentamicin Sigma-Aldrich (Auckland, NZ) and carbenicillin Sigma-Aldrich (Auckland, NZ) were added at concentrations of 100 to 300 μg/mL and 300 μg/mL, respectively.

ii) Isolation, analysis, and manipulation of DNA.

General cloning procedures were performed as described previously (16). DNA primers, deoxynucleoside triphosphates, and *Taq*, and Platinum *Pfx* polymerases were purchased from Life Technologies (Auckland, NZ). DNA sequences of new plasmid constructs were confirmed by DNA sequencing, according to the chain termination method, using an ABI 310 automatic sequencer.

iii) Construction of *single-*, *double-*, *and triple*-deletion mutants

Two regions of the *pelF* gene were amplified by using *Pfx* polymerase with primers PelF-N1, PelF-N2, PelF-C1, and PelF-C2. Both PCR products were hydrolyzed with *Bam*HI, ligated together, and inserted into vector pGEM-T Easy from Promega (Sydney, Australia), resulting in pGEM-T Easy::PelF-NC. A 1,100-bp fragment, containing the *aacC1* gene (encoding gentamicin acetyltransferase) flanked by two Flp recombinase target sites was released when vector pPS856 (17) was hydrolyzed with *Bam*HI. The 1,100-bp *Bam*HI fragment (*aacC1* gene) was inserted into the *Bam*HI site of plasmid pGEM-T Easy::PelF-NC, resulting in plasmid pGEM-TEasy::Δ*pelF* ΩGm

(where Gm is the gentamicin resistance cassette). A 1,949-bp Δ*pelF* ΩGm DNA fragment was amplified by *Pfx* polymerase using primers PelF-N1 and PelF-C2, and the corresponding PCR product was inserted into the *Sma*I site of vector pEX100T (17, 18), resulting in plasmid pEX100T::Δ*pelF* ΩGm. The gene-knockout plasmid was transferred into PAO1 via electroporation as previously described (19). Transformants were selected on LB medium containing 100μg of gentamicin/mL and subsequently plated on mineral salt medium (MSM) containing 300μg of gentamicin/mL and 5% (wt/vol) sucrose. Those cells able to grow on this selective medium will have emerged from double-crossover events. Gene replacement was confirmed via PCR with primers PelF XUP and PelF XDN. *E. coli* S17-1 was used to transfer the Flp recombinase-encoding vector pFLP2 (17) into *P. aeruginosa* PAO1 Δ*pelF Ω*Gm. Transfer of plasmid pFLP2 into recipient cells was confirmed by selecting carbenicilllin-resistant cells on the PIA medium containing carbenicillin (300μg/mL). These carbenicillin-resistant bacteria were cultivated on the PIA medium containing 5% (wt/vol) sucrose, and bacteria grown on this medium were analyzed by observing their sensitivity to gentamicin and carbenicillin. PCR with primers PelF XUP and PelF XDN was done to confirm the loss of the gentamicin resistance cassette. Consequently *P. aeruginosa* PAO1 Δ*pelF* and PDO300 Δ*pelF* were generated. Accordingly, plasmids pEX100T::Δ*alg8* ΩGm (20**)** and pEX100T::Δ*pslA* ΩGm (21) were used for the disruption of the *alg8* and/or *pslA* genes in *P. aeruginosa* PAO1. To generate Psl- and alginate-negative double mutants, single mutants PAO1 Δ*pslA* and PDO300 Δ*alg8* were transformed with pEX100T::Δ*pslA* ΩGm and pEX100T::Δ*alg8* ΩGm, respectively, and the resultant markerless PAO1 Δ*pslA* Δ*alg8* and PDO300 Δ*pslA* Δ*alg8* strains were generated as described above. To generate the respective Pel-deficient double and triple mutants pEX100T::Δ*pelF* ΩGm was transferred into markerless single and double mutants PAO1 Δ*alg8,* PAO1 Δ*pslA* (21), PDO300 Δ*alg8* (20), PDO300 Δ*pslA,* PAO1 Δ*pslA* Δ*alg8,* and PDO300 Δ*pslA* Δ*alg8*. The gentamicin resistance cassette was removed to obtain PAO1 Δ*alg8* Δ*pelF,* PAO1 Δ*pslA* Δ*pelF*, PDO300 Δ*alg8* Δ*pelF,* PDO300 Δ*pslA* Δ*pelF*, PAO1 Δ*pslA* Δ*alg8* Δ*pelF,* and PDO300 Δ*pslA* Δ*alg8* Δ*pelF*. All mutants were grown in liquid medium and 1ml of culture was stored in 7% DMSO at -80°C for future use.

iv) Complementation of isogenic *pelF* deletion mutant

For complementation of the isogenic knockout mutant *P. aeruginosa* PAO1 Δ*pslA* Δ*pelF*/pBBR1-MCS5::pelF was constructed. The *pelF* gene was amplified by PCR using the primers PelFEcRfor and PelFClaRev, together with chromosomal DNA from *P. aeruginosa* PAO1. The PCR product was inserted into the vector pGEM-T Easy. *Sal*I, one of the multiple cloning sites provided in vector pGEM-T Easy, was available upstream of the gene cloned into vector pGEM-T Easy. Thus, the *pelF* gene was restricted with *Sal*I and *Cla*I (for which a restriction site was available in the downstream primer), and inserted into the broad-host-range vector pBBR1-MCS5 restricted by *Sal*I and *Cla*I. In the resulting plasmid, pBBR1-MCS5::pelF, the *pelF* gene was arranged linear to and downstream of the *lac* promoter. The donor strain, *E. coli* S17-1, harboring pBBR1-MCS5::pelF was allowed to conjugate with PAO1 Δ*pslA* Δ*pelF*. Recipient PAO1 Δ*pslA* Δ*pelF* cells carrying pBBR1-MCS5::*pelF* were isolated by selection on PIA medium containing gentamicin (300μg/mL). To confirm the successful transfer of plasmid from donor to recipient strain, plasmids were isolated and digested with respective restriction enzymes.

v) Construction of broad-host-range promoter-probe vector pTZ110::P_{pel}

The broad-host-range promoter-probe vector pTZ110::P_{pel}, was constructed as follows. The primers PpelEcRfor and PpelBaRev and genomic DNA from *P. aeruginosa* PAO1 were used to amplify 1,000-bp upstream region of *pelA* comprising the putative promoter region of the *pel* gene operon. The PCR product was inserted into the vector pGEM-T Easy and transformed into *E. coli* Top10 cells. Hydrolysis with *Eco*RI (for which a site was present in the upstream primer) and *Bam*HI (for which a site was present in the downstream primer) yielded a 1000-bp putative promoter region P_{pel} which was subsequently cloned into the corresponding sites of pTZ110. In this plasmid the promoter region was upstream of *lacZ.* This plasmid was transferred to *P. aeruginosa* strains through conjugation via *E. coli* S17-1, and transconjugants were selected for on LB agar containing 300 μg/mL carbenicillin.

vi) β-Galactosidase assay

The β-Galactosidase activity was measured as described by Miller (22) and is expressed in Miller units (MU). The data presented below are the results obtained from three independent experiments. The standard deviation is indicated by error bars in the figures.

vii) Pellicle formation assay

Pellicle formation was assessed as previously described (5). Briefly, borosilicate glass tubes (18 mm by 150 mm) containing 10 mL of PI broth were inoculated with each mutant. Tubes were incubated without shaking at 37°*C*. Pellicle formation at the air-liquid interface after 4 days was assayed by visual inspection. Presence of pellicle was observed by seeing a thick layer of bacterial growth at the air-liquid interface. In absence of pellicle mutants did not show any such thick layer on air-liquid interface.

viii) Congo red binding assay

The assay to assess the ability of mutants to produce Pel polysaccharide was adapted from Spiers *et al.* (23). Each mutant was incubated in 2 mL of PI broth for 48 h at 37°C without shaking. The bacterial content along with polysaccharides produced by bacteria was collected by centrifugation, and supernatant was discarded. The pellet was washed with PI broth and transferred into 2-mL microfuge tubes. The pellet was re-suspended in 1 mL of 20 µg mL^{-1} Congo red in PI broth and incubated for 90 min while it was shaken. Bacterial content and bound Congo red were sedimented by centrifugation at 15,870 x *g* for 5 min. The supernatant was collected and the optical density at 490 nm (OD_{490}) was noted. The total Congo red percentage left in supernatant was measured.

ix) Solid surface attachment (SSA) assay

Attachment to a solid surface was assessed as previously described (16) and with some modifications. In brief, pertinent strains were grown overnight in PI broth and the OD at wavelength 600 nm was measured. An appropriate amount of overnight culture was added into PI broth to obtain 1:100 dilutions. Eight wells of a 96-well plate was inoculated with 100 µL of diluted culture of a particular strain, and incubated at 37°C

for 2 - 96 h. In order to remove planktonic/non-adherent bacteria at the end of each incubation time, plates were washed either using a vigorous or gentle washing procedure as previously described (5).

x) DNase treatment of biofilms

P. aeruginosa PAO1 Δ*pslA* Δ*alg8* and *P. aeruginosa* PAO1 Δ*pelF* Δ*alg8* were grown in flow cells for 96 h. DNase I from Sigma (St. Louise, MO) was dissolved in medium at a concentration of 500 µg/ mL and injected in to the flow cell. This was incubated for 30 min without flow. Flow was restored to 0.3 mL/min for 15 min, after which DNase-treated biofilm were stained as described below.

xi) Continuous-culture flow cell biofilms

For biofilm analysis, each mutant was grown in continuous-culture flow cells for 4 days at 37°C as previously described (24). The flow cells used in this study had dimensions of 4 mm by 40 mm by 1.5 mm. Each channel was filled with PI broth, inoculated with a total of 0.5 mL overnight culture of the respective mutant, containing approximately 2×10^9 cells per mL and incubated without flow for 4 h at 37°C. PI broth was then allowed to flow at a mean flow of 0.3 mL/min, corresponding to a laminar flow with a Reynolds number of 5. The flow cells were then incubated at 37°C for 96 h. Biofilms were stained using a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) and visualized using confocal laser scanning microscopy (CLSM; Leica SP5 DM6000B microscope). Images of *P. aeruginosa* and all of its mutants were captured using 63x objective lens. Images were analyzed using IMARIS (Bitplane, Inc) software.

xii) Quantitative analysis of biofilms using IMARIS image analysis software (Bitplane, Inc)

Biofilm appearance, biofilm volume, dead-to-live ratio, thickness, and compactness of biofilms were the parameters used to compare architectural differences of biofilms formed by the various mutants. To obtain volume per unit area ($\mu m^3/\mu m^2$), a ratio between total volume and total area covered by total biofilm was calculated. The compactness of the biofilm was assessed as total fluorescence per volume of biofilm. To

obtain the ratio between the dead to live cells in biofilm volume, the ratio between red and green fluorescence was calculated. In each biofilm, thickness of the base and microcolonies were measured separately, and standard deviations were calculated.

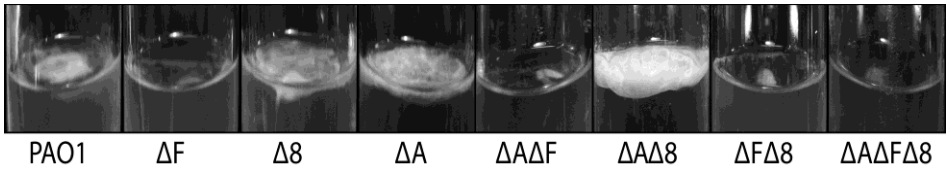
RESULTS

i) Generation of isogenic Δ*pelF* mutants deficient in the Pel polysaccharide production

Deletion of the *pelF* gene in PAO1 resulted in a Pel-deficient phenotype. Pellicle formation at the air-liquid interface was absent in the Δ*pelF* mutants, PAO1 Δ*pelF*, PAO1 Δ*pelF* Δ*alg8*, PAO1 Δ*pslA* Δ*pelF* and PAO1 Δ*pslA* Δ*pelF* Δ*alg8* (Figure.1A). The Congo red binding assay also showed that Pel-deficient mutants bind less Congo red, leaving most of it in the cell-free supernatant, whereas Pel polysaccharide-producing mutants PAO1, PAO1 Δ*alg8,* PAO1 Δ*pslA*, PAO1 Δ*pslA* Δ*alg8* showed increased binding of Congo red leaving less Congo red in the cell-free supernatant (Figure. 1B).

Similar Congo red staining results were shown for Pel-deficient mutants PDO300 Δ*pelF*, PDO300 Δ*alg8* Δ*pelF*, PDO300 Δ*pslA* Δ*pelF*, PDO300 Δ*pslA* Δ*alg8* Δ*pelF* (data not shown).

A



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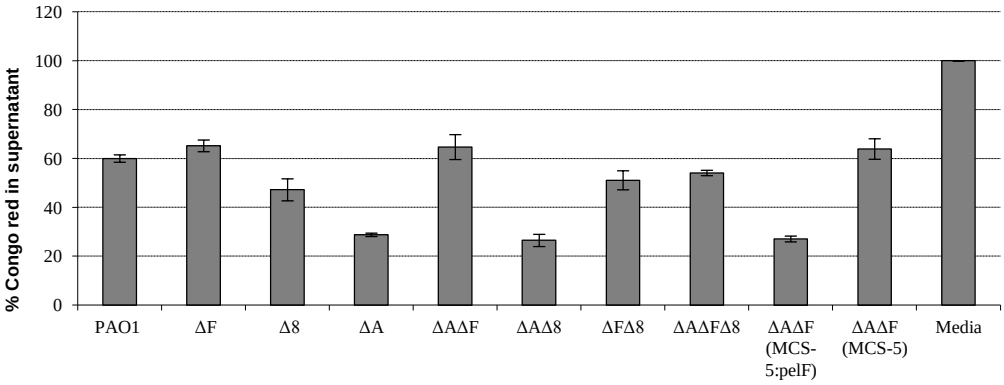


Figure 1. Assessment of Pel formation in various exopolysaccharide-deficient PAO1 mutants:_ΔF, PAO1 Δ*pelF*; Δ8, PAO1 Δ*alg8*; ΔA, PAO1 Δ*pslA,* ΔAΔF; PAO1 Δ*pslA* Δ*pelF,* ΔAΔ8; PAO1 Δ*pslA* Δ*alg8*, ΔFΔ8; PAO1 Δ*alg8* Δ*pelF*, ΔAΔFΔ8; PAO1 Δ*pslA* Δ*pelF* Δ*alg8*. (A) Pellicle formation at air-liquid interface when each mutant was grown in 10 mL of PI medium for 4 days as a static biofilm. (B) Congo red binding assay. All strains grown as static biofilms for 4 days were mixed with 20 µg/ml Congo red and left for 90 min while it was shaken. The biomass was sedimented, and unbound Congo red was detected at 490 nm. The percentage of Congo red left in the supernatant is shown here are the mean values of three independent assays and bars represent standard deviations.

ii) Complementation of the Δ*pelF* mutant

To confirm that disruption of the *pelF* gene had no polar effect on genes downstream of *pelF* within the *pel* operon, plasmid pBBR1-MCS5::*pelF* was constructed and transformed into *P. aeruginosa* PAO1 Δ*pslA* Δ*pelF*. Since the lack of Pel production of the *pelF* mutant was particular obvious in the Psl-negative background, the double mutant PAO1 Δ*pslA* Δ*pelF* was used for complementation experiments. The mutant PAO1 Δ*pslA* Δ*pel*F harboring plasmid pBBR1-MCS5::pelF was restored in its ability to produce Pel, which was shown by the formation of a pellicle at the air-liquid interface and increased Congo red binding (Figure.1B).

iii) Generation of various isogenic deletion mutants deficient in the production of various exopolysaccharides

To study the synergistic or antagonistic effects of the three polysaccharides on biofilm formation, single, double, and triple mutants of strains PAO1 and PDO300 were generated. Previously, a PAO1 Δ*pslA* mutant was generated and characterized and was shown to be deficient in attachment to solid surface at early stage of biofilm formation (21). PAO1 Δ*pslA* was used to generate Psl/alginate-, Psl/Pel-, and Psl/alginate/Pel-deficient mutants. The resultant mutants, PAO1 Δ*pslA* Δ*alg8,* PAO1 Δ*pslA* Δ*pelF,* and PAO1 Δ*pslA* Δ*alg8* Δ*pelF,* respectively, showed impaired attachment in the SSA assay (Figure. 2A). PDO300 Δ*pslA,* PDO300 Δ*pslA* Δ*alg8,* PDO300 Δ*pslA* Δ*pelF,* and PDO300 Δ*pslA* Δ*alg8* Δ*pelF* strains generated in this study also showed impaired solid surface attachment (see Figure. S1 in the supplementary material).

PDO300 Δ*alg8* has already been previously characterized as deficient in alginate production (20). All *alg8*-knockout mutants of PDO300, PDO300 Δ*pslA* Δ*alg8,* PDO300 Δ*alg8* Δ*pelF,* and PDO300 Δ*pslA* Δ*alg8* Δ*pelF,* were non-mucoid when grown on PIA plates and showed no alginate production when they were assessed for alginate quantification (see Table S2 in the supplementary material). Isogenic mutants PAO1 Δ*alg8,* PAO1 Δ*pslA* Δ*alg8,* PAO1 Δ*alg8* Δ*pelF,* and PAO1 Δ*pslA* Δ*alg8* Δ*pelF* were generated as outlined in Table S1 in the supplementary material. An overview of all strains used and generated in this study and their exopolysaccharide biosynthesis-relevant genotype and phenotype are presented in Table S1.

Table 1. An overview of exopolysaccharides produced by mutants used in this study.

Strains	Exopolysaccharides Produced by strains[a]		
	Alginate	Pel	Psl[b]
PAO1	+	+	+
PAO1Δ*pelF*	+	-	+
PAO1Δ*alg8*	-	++	+
PAO1Δ*pslA*	+	+++	-
PAO1Δ*pslA*Δ*pelF*	+	-	-
PAO1Δ*pslA*Δ*alg8*	-	+++	-
PAO1Δ*pelF*Δ*alg8*	-	-	+
PAO1Δ*pslA*Δ*pelF*Δ*alg8*	-	-	-
PDO300	+++	+	+
PDO300Δ*pelF*	++++	-	+
PDO300Δ*alg8*	-	++	+
PDO300Δ*pslA*	++	+++	-
PDO300Δ*pslA*Δ*pelF*	++++	-	-
PDO300Δ*pslA*Δ*alg8*	-	+++	-
PDO300Δ*pelF*Δ*alg8*	-	-	+
PDO300Δ*pslA*Δ*pelF*Δ*alg8*	-	-	-

a -; Polysaccharide production not detectable, +; Polysaccharides production detectable, with increased numbers of plus signs indicating increased relative production.

b Psl quantification was not done due to unavailability of a specific and differentiating detection method. However, its production was indicated by determining the total exopolymers produced by the alginate/Pel-deficient mutant.

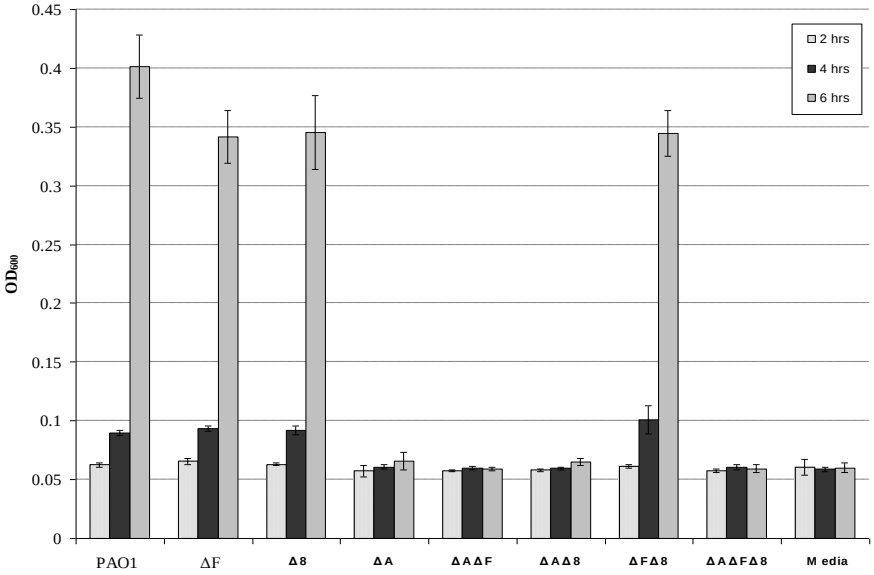
iv) Effect of anti-sigma factor MucA, on transcription of the *pel* and *psl* operons

The measured β-galactosidase activity was used to deduce the respective promoter activities. The pel promoter in PAO1 and PDO300 mediated a β-galactosidase activity of 683 ± 158 and 564 ± 68 Miller units, respectively. The psl promoter in PAO1 and PDO300 mediated a β-galactosidase activity of 2,704 ± 477 and 1,923 ± 183 Miller units, respectively.

v) Analysis of the role of the various exopolysaccharides with respect to attachment to solid surfaces

An SSA assay was conducted to observe the attachment of cells at initial and mature stages of biofilm development. After 2, 4, and 6 h of incubation, the strains producing Psl (PAO1, PAO1 Δ*pelF*, PAO1 Δ*alg8*, PAO1 Δ*pelF* Δ*alg8*) showed increased attachment compared to Psl-negative mutants. After 4 days of growth, Psl-negative, Pel-producing mutants PAO1Δ *pslA* and PAO1 Δ*pslA* Δ*alg8* showed increased attachment compared to Pel-deficient mutants. Results are summarized in Figure. 2 (Please find the figure on page 78). Similar results were found for PDO300 and derived isogenic mutants (see Figure. S1 in Supplementary material).

A



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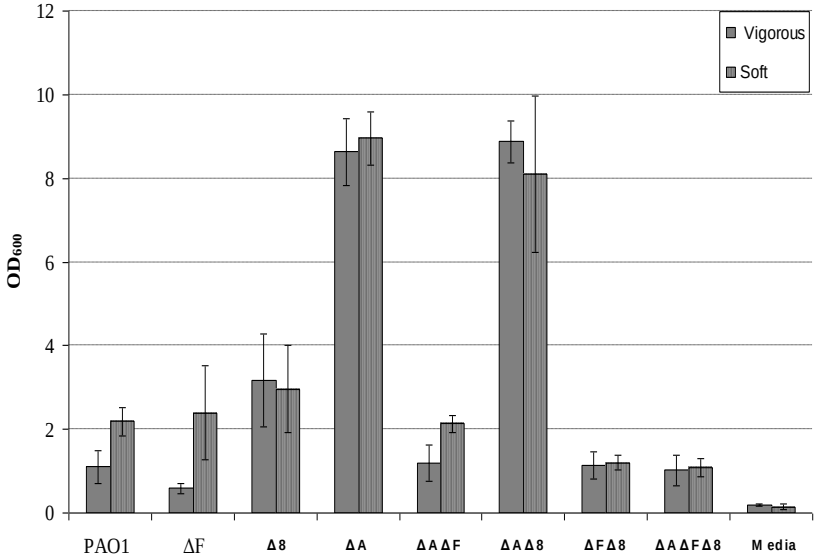

Figure 2. Attachment of various *P. aeruginosa* PAO1 strains to solid surface. The SSA was used to assess the impact of various exopolysaccharide deficiencies on attachment. PAO1; *P. aeruginosa* PAO1, ΔF; PAO1Δ*pelF*, Δ8; PAO1Δ*alg8*, ΔA; PAO1Δ*pslA*, ΔAΔF; PAO1Δ*pslA*Δ*pelF*, ΔAΔ8 ; PAO1Δ*pslA*Δ*alg8*, Δ8ΔF; PAO1 Δ*alg*8*pelF*, ΔAΔFΔ8; PAO1Δ*pslA*Δ*pelF*Δ*alg8*, Media, un-inoculated PI medium as a control; OD_{600}, optical density at 600 nm. (A) Differences during early attachment phase; (B) differences between loosely and tightly attached 4 days old biofilms (adherent biofilms after soft and vigorous washing, respectively). Values and error bars represent the averages and standard deviations, respectively, for twenty four independent replicates.

vi) Analysis of biofilms

Representative images of the biofilms of respective exopolysaccharide-negative mutants of *P. aeruginosa* were obtained using CLSM (Figure. 3 on page 80 and Figure.S2 and S3 in the supplementary material). The CLSM stack images were reconstructed into three-dimensional (3D) images using IMARIS software. The results showed that PAO1 Δ*alg8* was not able to form mushroom-like structures. Mutants including PAO1 Δ*pelF* and PAO1 Δ*pelF* Δ*alg8,* capable of producing Psl and alginate or only Psl, respectively, formed mushroom-like structures. Interestingly, PAO1 Δ*pelF*, which produces Psl and alginate, formed mushroom- like structures. Interestingly, PAO1 Δ*pelF*, which produces Psl and alginate, formed mushroom-like structures with higher density of living cells than PAO1 Δ*pelF* Δ*alg8* biofilms, which additionally lacked alginate and formed mushroom-like structures with the caps almost devoid of living cells (Figure. 3C and E). PAO1 Δ*pslA* and PAO1 Δ*pslA* Δ*alg8* were still able to form biofilms, but these biofilms were flat and much more compact than the biofilms formed by all other studied mutants, and both live and dead cells were present in these biofilms (Figure. 3D and F). Interestingly, it was observed that PAO1 Δ*pslA* Δ*pelF* and PAO1 Δ*pslA* Δ*pelF* Δ*alg8* were not able to form any biofilm after 96 h, and only cells spread around were observed (see Figure. S2 in the supplementary material). Results similar to those obtained for the various PAO1 mutants were obtained when the biofilms of the respective PDO300 mutants were analyzed (see Figure S3A to G in the supplementary material) except for PDO300 Δ*pelF,* which could not be assessed due to the excessive formation of exopolymeric material and the subsequent blocking of the flow cell. The levels of alginate produced by this strain were found to be about 10-fold increased compared with those produced by parent strain PDO300 (see Table S2 in the supplementary material). Although mutant PDO300 Δ*pelF* Δ*pslA* showed a similar overproduction of alginate, biofilms could still be grown in flow cells without blockage.

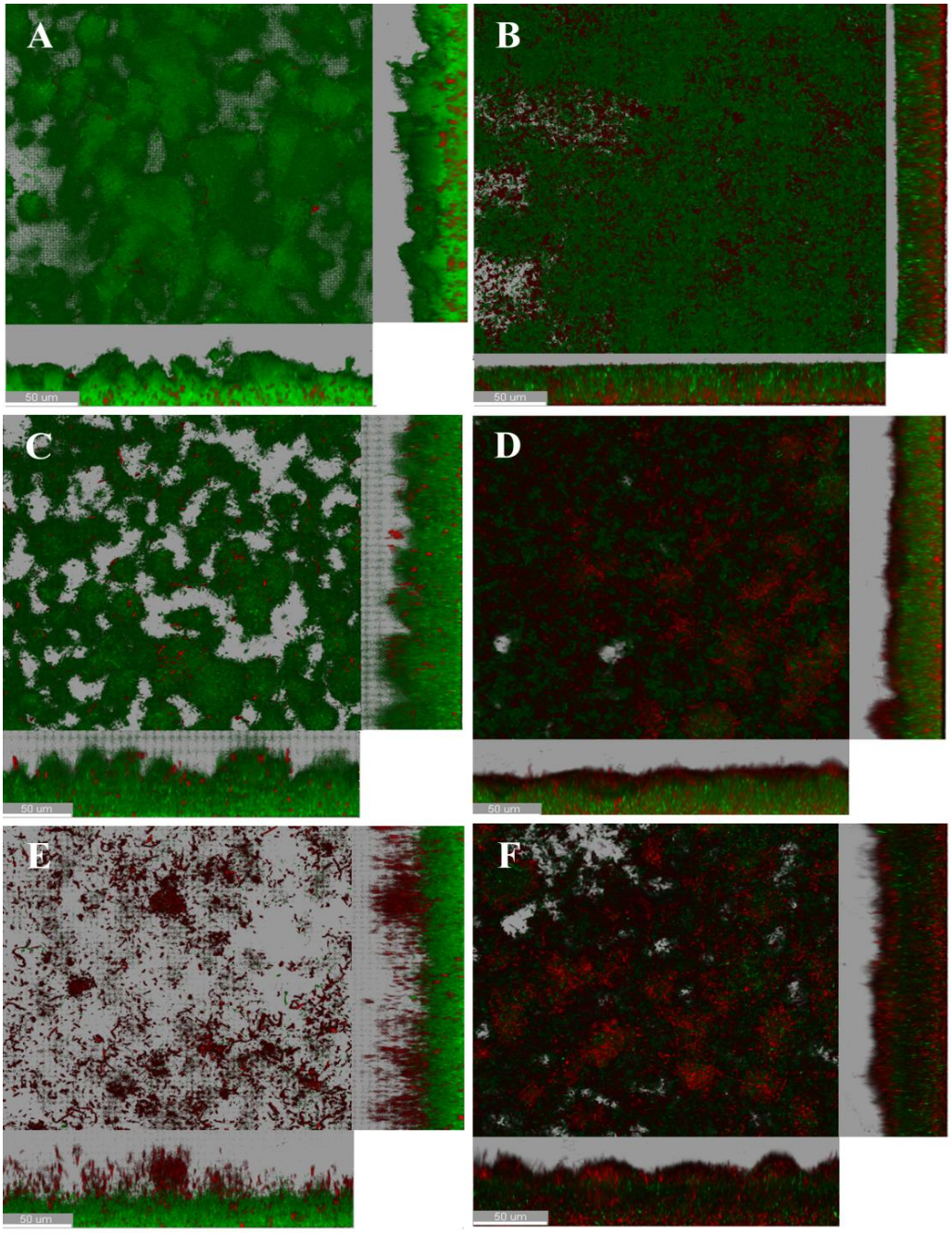

Figure 3. Confocal laser scanning microscopic images of *P. aeruginosa* biofilms (grown in a continuous-culture flow cell for 4 days). XY (central), XZ (bottom) and YZ (left) plans of each image are shown. A; *P. aeruginosa* PAO1 wild type, B; PAO1 Δ*alg8,* C; PAO1Δ*pelF*, D; PAO1Δ*pslA*, E; PAO1 Δ*alg8*Δ*pelF*, F; PAO1Δ*pslA*Δ*alg8*. Red colour represents the dead cells/eDNA stained by Propidium Iodide and green colour represents the live cells stained by cell-permeant nuclic acid stain SYTO-9.

vii) DNase treatment of mutants PAO1 Δ*pslA* Δ*alg8* and PAO1 Δ*pelF* Δ*alg8*

The biofilms of the PAO1 Δ*pslA* Δ*alg8* and PAO1 Δ*pelF* Δ*alg8* strains showed increased levels of red fluorescence, indicating dead cells and eDNA. To assess the role of eDNA in biofilms, DNase treatment was conducted. This resulted in almost complete removal of red fluorescent structures (Figure. 4A to D), as was indicated by a decrease of the red fluorescence-to-green fluorescence ratio from 1.08 to 0.45 in the case of PAO1 Δ*pslA* Δ*alg8* and 0.8 to 0.2 in the case of PAO1 Δ*pelF* Δ*alg8.*

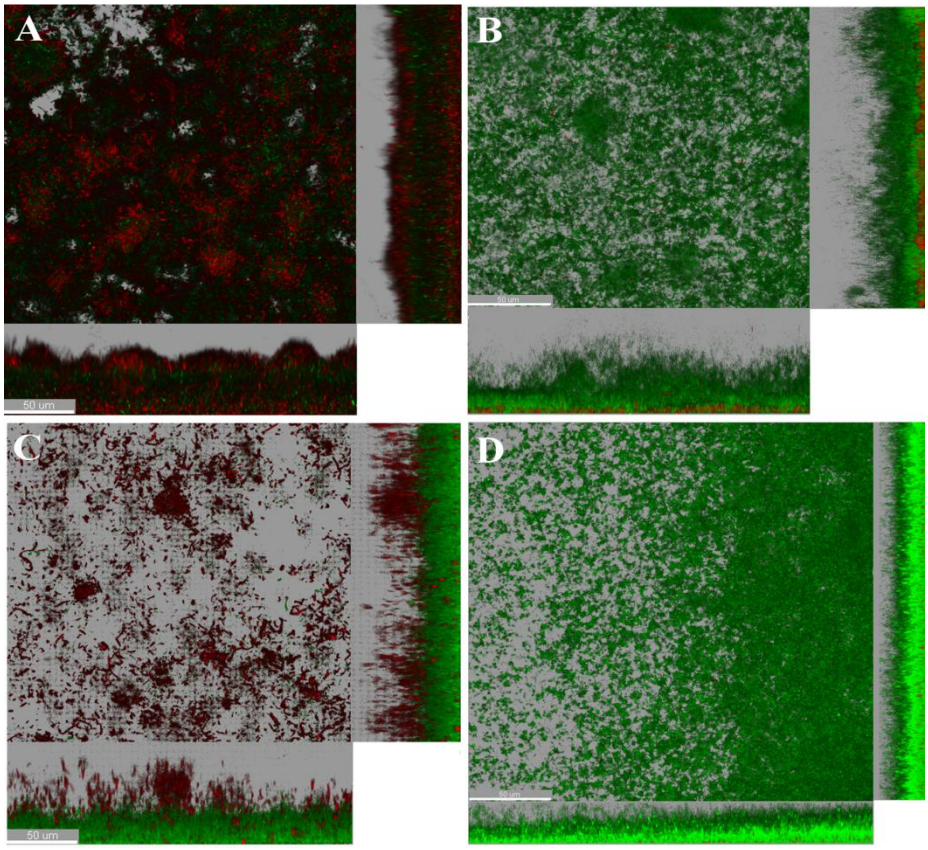

Figure 4 DNase treatment of high amount of extracellular DNA producing mutants biofilms. A; Images of PAO1Δ*pslA*Δ*alg8* without DNase treatment, B; Images of PAO1Δ*pslA*Δ*alg8* after DNase treatment*,* C*;* Images of PAO1Δ*pelF*Δ*alg8* without DNase treatment, D; Images of PAO1Δ*pelF*Δ*alg8* after DNase treatment.

vii) Quantitative analysis of biofilms

The biofilm volume, dead-to-live ratio, thickness, and compactness of biofilms were assessed using IMARIS software. To obtain the volume per area ratio the total volume ($x \times y \times z = \mu m^3$) and total area ($x \times y = \mu m^2$) covered by each biofilm was measured. The highest volume-to-area ratio was found for PAO1 and PAO1 Δ*pslA* Δ*alg8* biofilms. PAO1 Δ*alg8* has showed the lowest volume-to-area ratio (Table 2).

Table 2 Characteristics of biofilms formed by *P. aeruginosa* mutants

Biofilm forming strains	Volume ($\mu m^3/\mu m^2$)	Dead:Live Ratio*
PAO1	47.21	0.33
PAO1Δ*pelF*	35.63	0.53
PAO1 Δ*alg8*	28.91	0.41
PAO1Δ*pslA*	25.09	0.65
PAO1Δ*pslA*Δ*alg8*	49.37	1.08
PAO1Δ*pelF*Δ*alg8*	42.57	0.81

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

The biofilms formed by PAO1Δ*pslA,* PAO1 Δ*pslA* Δ*alg8,* and PAO1 Δ*alg8* were found to be more compact than the other biofilms. Intensity of fluorescence per unit biofilm volume was used to compare the compactness of biofilms. All biofilms formed by Pel-producing PAO1, PAO1 Δ*alg8*, PAO1 Δ*pslA, and* PAO1 Δ*pslA* Δ*alg8* showed 1.83×10^3, 1.43×10^3, 3.75×10^3 , and 1.79×10^3 relative light intensity per μm^3, respectively, which is greater than that of biofilms formed by Pel-deficient mutants PAO1 Δ*pelF* and PAO1 Δ*pelF* Δ*alg8* showing 1.07×10^3 and 8.82×10^2 relative light intensity per μm^3, respectively. The ratio of dead cells to live cells in the various biofilms showed that in the absence of alginate, biofilms contain more dead cells and extracellular DNA. Dead to live cell ratios in biofilms produced by alginate-negative mutants PAO1 Δ*alg8*, PAO1 Δ*pslA* Δ*alg8,* and PAO1 Δ*pelF* Δ*alg8* were higher than those in alginate-producing PAO1, PAO1 Δ*pslA*, and PAO1 Δ*pelF* biofilms (Table 2).

Biofilms formed by all strains contained a base layer of various heights. Psl-producing PAO1**,** PAO1 Δ*pelF*, and PAO1 Δ*pelF* Δ*alg8* formed structured biofilms having a base layer one-fourth to one-fifth of the total height of the microcolony, and the microcolony could be clearly differentiated into stalk and mushroom-like cap structures. On the other hand, Psl-deficient mutants PAO1 Δ*pslA* and PAO1 Δ*pslA* Δ*alg8* formed unstructured flat biofilms, where the only discernible features were short dome-like structures. Interestingly, PAO1 Δ*alg8*, which produces Psl and Pel, showed an unstructured flat biofilm similar to that of Pel-overproducing, Psl-deficient mutants (Figure. 5).

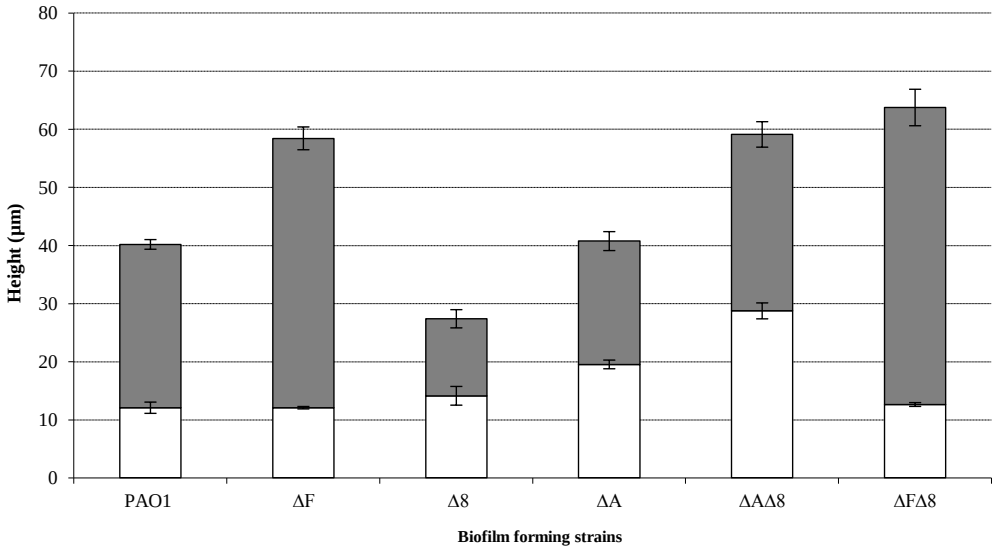

Figure 5. Height of microcolonies and base layer of each biofilm-forming mutant measured from 3D pictures of biofilms grown for 96 h. White, height of base of the biofilm. ΔF; PAO1 Δ*pelF*, Δ8; PAO1 Δ*alg8*, ΔA; PAO1 Δ*pslA*, ΔAΔ8; PAO1 Δ*pslA* Δ*alg8*, ΔFΔ8; PAO1 Δ*alg8* Δ*pelF*.

DISCUSSION

In this study the role of single exopolysaccharides and various combinations of exopolysaccharides in biofilm formation by *P. aeruginosa* was investigated. Three exopolysaccharides, Psl, Pel and alginate, have been described to be produced by *P. aeruginosa* as well as have been found to play a role in biofilm formation and biofilm architecture (25). To elucidate the role of each exopolysaccharide, key biosynthesis genes required for the production of the respective exopolysaccharide were disrupted independently and in various combinations. Hence, isogenic deletion mutants of strains PAO1 and its *mucA*-negative derivative PDO300 deficient in the production of one or more exopolysaccharides were generated for comparative analysis.

Previous work had established that knocking out *alg8* from *P. aeruginosa* results in the complete loss of alginate production, as shown by the lack of alginate and uronic acids in the culture supernatants (26). Similarly, deletion of *pslA* had been shown to abolish Psl production, which impaired surface attachment (21). Hence, mutants deficient in alginate and Psl were generated in this study by knocking out *alg8* and *pslA*, respectively. Previously, it was shown that a third exopolysaccharide, Pel, is produced by *P. aeruginosa* and was found to be important for the formation of static biofilms by various strains (5). Seven genes contained in a single operon (*pelA* to *pelG*) are required for production of the Pel exopolysaccharide (5, 33).The *pelF* sequence analysis suggested that it encodes a glycosyltransferase, presumably involved in the polymerization of the Pel exopolysaccharide (5). Deletion of *pelF* from *P. aeruginosa* PAK genome significantly reduced biofilm formation compared to that by the parent strain (26). The inability of mutants to produce Pel had been characterized by a lack of pellicle formation at the air-liquid interface and reduced Congo red binding (5, 11). Here, *pelF* was deleted from PAO1 and PDO300 in order to generate isogenic mutants, deficient in Pel exopolysaccharide biosynthesis. All *pelF* mutants showed reduced Congo red binding and the absence of pellicle formation. Although the specificity of this Congo red binding assay has not been established, it has been widely used as an indicator of Pel production (5, 32, 33). Our results showed that all mutants deficient in pellicle formation at the air liquid-interface also showed reduced Congo red binding (Figure. 1).

Previous studies showed that Psl is involved in initial attachment of cells (2, 6, 10, 21) and Pel plays a role in the later stages of biofilm maturity (6). Our results were consistent with these findings. Mutants deficient in Psl showed reduced crystal violet staining of static biofilms at an early stage of biofilm development. Furthermore, neither alginate nor Pel appeared to compensate for this deficiency (Figure. 2A and B). However, in mature biofilms, increased crystal violet staining of the Pel-overproducing mutant PAO1 Δ*pslA* Δ*alg8* suggested that Pel at later stages of biofilm development mediated entrapment of more cells (Figure. 2). Since Pel has been shown to contribute to cell-to-cell interactions in *P. aeruginosa* PA14 (27), increased Pel production should further increased cell-cell interactions. PA14 is a naturally Psl-deficient strain, and the Psl-deficient mutants generated here are similar to PA14 in this respect. Therefore, it is conceivable that increased crystal violet staining resulted from an increased number of bacterial cells in the biofilm held together by the excessive Pel produced by these mutants.

Here it was demonstrated that mutants lacking alginate production showed an increased ratio of dead to live cells (Table 2, Figure. 3). However, propidum iodide also stains eDNA (28). PAO1 Δ*pslA* Δ*alg8* and PAO1 Δ*pelF* Δ*alg8* biofilms showed extensive surface structures predominantly composed of eDNA, as was shown by DNase treatment (Figure. 4). Previous studies suggested that eDNA plays an important role in biofilms as a cation-chelating and antibiotic resistance-inducing matrix component (14, 28). Since large amounts of eDNA were only observed in mutants lacking alginate production, increased cell death might have led to more eDNA being released functionally and structurally replacing the polyanionic alginate.

The role of alginate for entrapping live cells in the cap of the mushroom-like structure was underpinned by the observation that the caps in biofilms formed by PAO1 Δ*pelF* Δ*alg8* (produces only Psl) were almost devoid of live cells, whereas those formed by Psl- and alginate-producing strains PAO1, PAO1 Δ*pelF* (Figure. 3), and PDO300 (see Figure. S3A in the supplementary material) showed a high density of live cells in the cap region. This suggested that the caps of the mushroom-like structures are not made up of only Psl but that alginate plays an important role in retaining live cells and/or supporting the viability of cells in these caps.

Interestingly, analysis of biofilms formed by PAO1 Δ*pslA* Δ*alg8* suggested that Pel and eDNA together with live bacterial cells constituted a connected meshwork showing increased cell-to-cell interactions and, hence increased compactness of the biofilms (Figs. 3 and 4). This is consistent with the findings of a study that suggested that increased Pel production enhanced cell-to-cell interactions as well as increased biomass of biofilms (4). This compactness might be the reason why even when Psl is additionally produced (PAO1 Δ*alg8*) elevated mushroom-like structures were not formed (Figure. 5). The increase in volume and height (Figure. 5) and the decrease in compactness (Figure. 3C and E) of biofilms produced by Pel-deficient mutants PAO1 Δ*pelF* and PAO1 Δ*pelF* Δ*alg8* compared to those produced by the parent strain provided further evidence that Pel contributes to the compactness of the biofilm. Unlike Pel biofilms of PAO1 Δ*pelF* Δ*alg8* with Psl along with eDNA did not show such cell-to-cell interactions. This is in accordance with the findings of previous studies, which suggested that Psl and eDNA in a biofilm are not located in close vicinity (8).

Interestingly, the Psl-negative mutant showed more Pel production in static biofilms (Figure. 1) and the Pel-deficient mutant showed increased alginate production when it was grown on solid media (see Table S2 in the supplementary material) compared with the respective wild-type strains. This could be due to competition of the biosynthesis pathways of the Pel, Psl, and alginate with respect to metabolic precursors. Polymer biosynthesis pathways in *P. aeruginosa* had been suggested to be competitive with respect to common precursors (22, 24). However, the possibility that the absence of one or two exopolysaccharides impacts at the level of the regulation of biosynthesis of the still produced exopolysaccharide or exopolysaccharides cannot be excluded.

To assess how the anti-sigma factor MucA impacts the ability to produce Pel and Psl at the transcriptional level, the activities of the *pel* and *psl* promoters was assessed in the PAO1 and PDO300 strains, respectively. This showed that the lack of MucA did not significantly impact the *pel* promoter activity but decreased the *psl* promoter activity by about 30%. It is known that a deficiency of MucA results in increased alginate production and transcriptional activation of the alginate operon (29). Here it seemed that this increase in alginate production and/or the unleashed AlgU had a negative effect on the transcriptional activity of the *psl* operon. This suggested some regulatory cross talk between the regulations of the biosynthesis of various

exopolysaccharides. Further investigations are required to shed further light on regulation of polysaccharide biosynthesis.

Overall, this study showed that thick-structured biofilms are still formed by mutants producing one or two polysaccharides, except when only alginate is produced. Only when neither Psl nor Pel (PAO1 Δ*pslA* Δ*pelF*) was produced, cells were not able to form biofilms (Figure. 4G and H). Experimental evidence that the deficiency in one or two exopolysaccharides, enhanced production of the remaining exopolysaccharides or exopolysaccharide, respectively, was provided (Figure. 1; see Table S2 in the supplementary material). These data shed new light on how the three polysaccharides and eDNA interactively contribute to *P. aeruginosa* biofilm formation and architecture.

Acknowledgments

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SUPPLEMENTARY MATERIAL

Table S1. Bacterial strains, plasmids, and oligonucleotide used in this study

Strains, plasmids, or oligonucleotide	Description or sequence (5´-3´)	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototrophic wild-type non mucoid strain	(29)
PDO300	*mucA22* isogenic mutant derived from PAO1	(15)
PAO1Δ*pelF*	Markerless, isogenic *pelF* deletion mutant derived from PAO1	This study
PAO1Δ*pslA*	Markerless, isogenic *pslA* deletion mutant derived from PAO1	(21)
PAO1Δ*alg8*	Markerless, isogenic *alg8* deletion mutant derived from PAO1	This study
PAO1Δ*pslA*Δ*pelF*	Markerless, isogenic *pslA* and *pelF* deletion double mutant derived from PAO1Δ*pslA*	This study
PAO1Δ*pslA*Δ*alg8*	Markerless, isogenic *pslA* and *alg8* deletion mutant derived from PAO1Δ*pslA*	This study
PAO1Δ*pelF*Δ*alg8*	Markerless, isogenic and *alg8* deletion mutant derived from PAO1	This study
PAO1Δ*pslA*Δ*pelF*Δ*alg8*	Markerless, isogenic *pslA* , *pelF*, and *alg8* deletion mutant derived from PAO1Δ*pslA*	This study
PDO300Δ*pelF*	Markerless, isogenic *pelF* deletion mutant derived from PDO300	This study
PDO300Δ*pslA*	Markerless, isogenic *pslA* deletion mutant derived from PDO300	This study
PDO300Δ*alg8*	Markerless, isogenic *alg8* deletion	(20)

	mutant derived from PDO300	
PDO300Δ*pslA*Δ*pelF*	Markerless, isogenic *pslA* and *pelF* deletion mutant derived from PDO300	This study
PDO300Δ*pslA*Δ*alg8*	Markerless, isogenic *pslA* and *alg8* deletion mutant derived from PDO300Δ*alg8*	This study
PDO300Δ*pelF*Δ*alg8*	Markerless, isogenic *pelF* and *alg8* deletion mutant derived from PDO300Δ*alg8*	This study
PDO300Δ*pslA*Δ*pelF*Δ*alg8*	Markerless, isogenic *pslA* , *pelF,* and *alg8* deletion mutant derived from PDO300Δ*alg8*	This study
E.coli.		
TOP10	*F-mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*	Invitrogen
Plasmids		
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	(30)
pBBR1MCS-5:pelF	*Sal*1-*Cla*1 fragment comprising gene *pelF* inserted into vector pBBR1MCS-5	This study
pEX100T	Ap^r Cb^r, gene replacement vector containing *sacB* gene for counterselection	(18)
pEX100T:Δ*pelF* Gm	Ap^r Cb^r Gm^r; vector pEX100T with *Sma*I-inserted *pelF* deletion construct	This study
pEX100TΔ*alg8*Gm	Ap^r Cb^r Gm^r; vector pEX100T with *Sma*I-inserted *alg8* deletion construct	(20)
pEX100TΔ *pslA* Gm	pEX100T containing GM cassette flanked by 5' and 3' ends of the *pslA* gene	(21)
pPS856	Ap^r Gm^r; source of 1,100-bp *Bam*HI	(17)

	fragment comprising *aacC1* gene flanked by FRT signal sequences	
pPFLP2	Ap^r Cb^r; broad-host-range vector encoding Flp recombinase	(17)
pTZ110	Broad-host-range *lacZ* fusion vector	(31)
pTZ110::P_{psl}	pTZ110 containing *Eco*RI/*Bam*HI PCR fragment of *psl* promoter	(21)
pTZ110::P_{pel}	pTZ110 containing *Eco*RI/*Bam*HI PCR fragment of *pel* promoter	This study
Oligonucleotides		
PelFN1	AAAACCCGGGATGACCGAACAC ACCGCTCCGAC	
PelFN2	AAAGGATCCTGCTGTGGAGAAA GTCCTCGCG	
PelFC1	AAAGGATCCGTGAAGTTCCTCG GTTTCCGTC	
PelFC2	AAAACCCGGGTCATGCAATCTC CGTGGCTTCG	
PelF XUP	ACATGCTGCAACGGCCGCCCT	
PelF XDN	TAGGCGCGCAGGGTCGCCGTA	
PslA XUP	GGACTGCCCGTGATCGGCAGAG	
PslA XDN	CCGGAAACGATGCAGGGGATCA	
Alg8 XUP	GCGTCGAGGCCAAGGTCCC	
Alg8 XDN	CCTGGCGTTGTCCGTAGTCG	
pelF-EcRfor	AAAGAATTCGGCCCTGGCGAGA TACTGGACATGACCGAACAC	
pelF-ClaRev	AAAATCGATTCATGCAATCTCCG TGGCTTCGC	
Ppel- EcRfor	AAAGAATTCGCGCTCGCACGCC GTTACGGCAC	
Ppel- BaRev	AAAGGATCCGCCCAGCCTACGC GGCAGGGTCG	

Table S2 Alginate quantification of various *P. aeruginosa* PDO300 mutants

Mutants	Total polymer production (g/g CDM) ± SD[c]	Alginate Production (g/g CDM) ± SD [a]
PDO300	0.79 ± 0.00	0.225[c]±0.07[c]
PDO300 Δ*pelF*	5.05 ± 0.53	2.23±0.31
PDO300 Δ*alg8*	0.09 ± 0.02	ND
PDO300Δ*pslA*	0.41 ± 0.06	0.14±0.02
PDO300Δ*pslA*Δ*pelF*	3.68 ± 0.76	1.97±0.23
PDO300Δ*pslA*Δ*alg8*	0.97 ± 0.17	ND
PDO300Δ*pelF*Δ*alg8*	0.35 ± 0.01	ND
PDO300Δ*pslA*Δ*pelF*Δ*alg8*	0.52 ± 0.02	ND

[a] CDM, cell dry mass

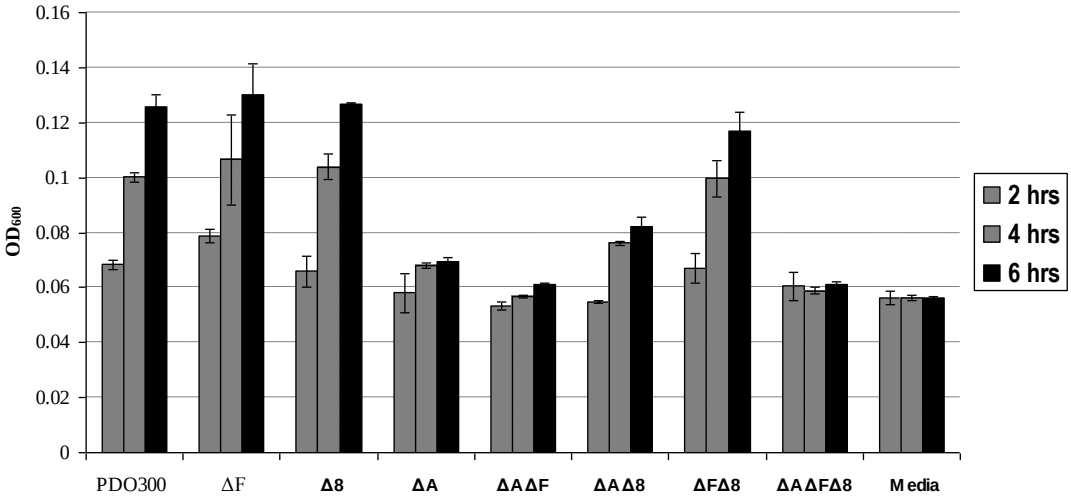
[b] SD, Standard deviation. Results represent the results of experiment done in duplicate.

[c] As obtained from isopropanol precipitation of respective cell-free supernatants

Uronic acid assay and EPS purification

Alginate concentrations were quantified by a modification of the Blumenkrantz and Asboe-Hansen protocol (32), using purified *P. aeruginosa* PDO 300 alginate (100% [wt/wt] uronic acid content) as standard, as previously described (20). Quantification of polymers and dry cell mass was determined from isopropinol precipitates of supernatant fraction of 72 h grown mutants on Pseudomonas isolation agar (PIA).

A



B

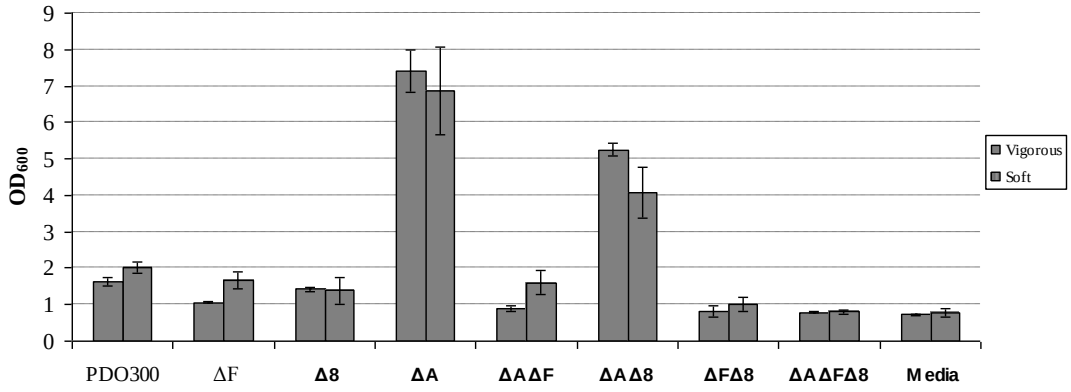


Figure S1. Attachment of various PDO300 strains to solid surface. The SSA was used to assess the impact of various exopolysaccharide deficiencies on attachment. PDO300; PDO300, ΔF; PDO300Δ*pelF*, Δ8; PDO300 Δ*alg8*, ΔA; PDO300Δ*pslA*, ΔAΔF; PDO300Δ*pslA*Δ*pelF*, ΔAΔ8; PDO300Δ*pslA*Δ*alg8*, Δ8ΔF; PDO300 Δ*alg*Δ*8pelF*, ΔAΔFΔ8; PDO300Δ*pslA*Δ*pelF*Δ*alg8*, Media; uninoculated PI broth control, OD_{600}; optical density at 600 nm. (A) Differences during early attachment phase, (B) differences between loosely and tightly attached 4 days old biofilms (adherent biofilms after soft and vigorous washing, respectively). Values and error bars represent the averages and standard deviations, respectively, for eighteen independent replicates.

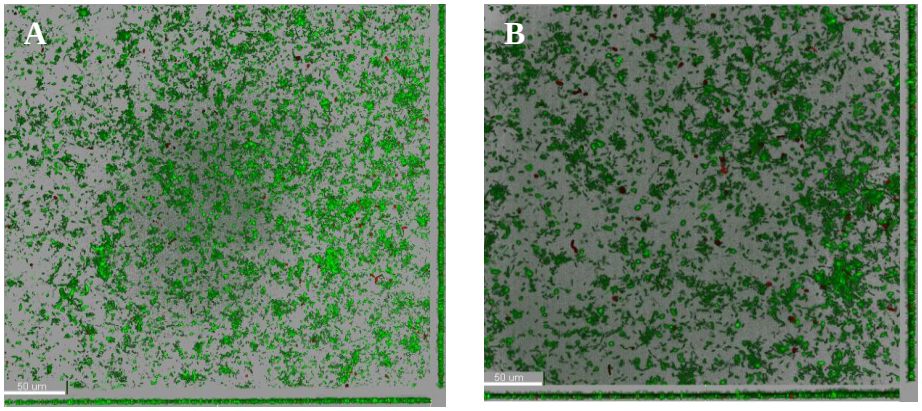


Figure S2. Confocal laser scanning microscopic images of PAO1Δ*pslA*Δ*pelF* (A) and PAO1Δ*pslA*Δ*pel*Δ*alg8* (B) grown in a continuous-culture flow cell for 4 days. XY (central), XZ (bottom) and YZ (left) plans of each image are shown. Both these mutants were unable to form multilayer biofilms. Single layer of cells can be seen in figures.

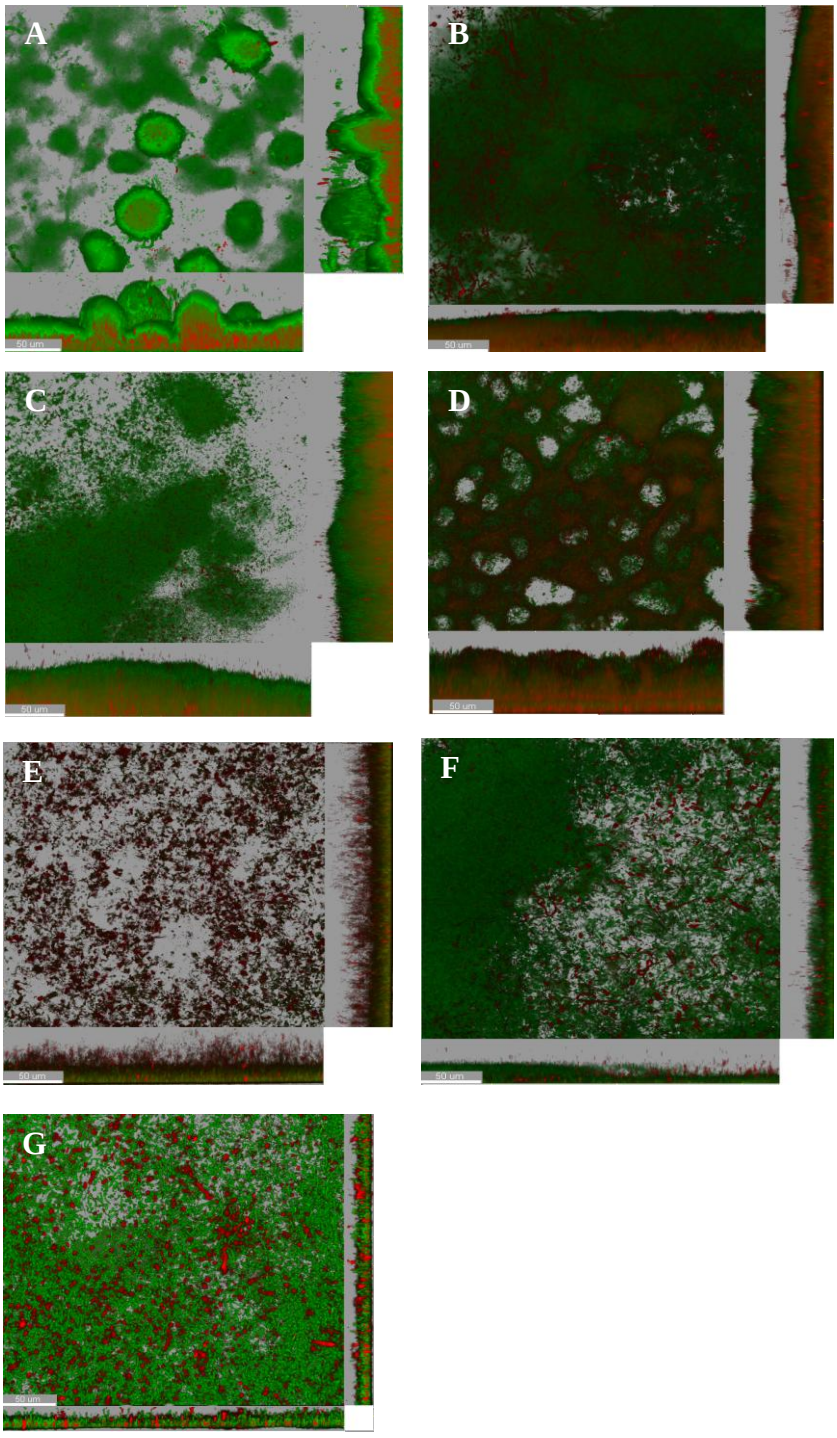


Figure-S3 Confocal laser scanning microscopic images of PDO300 biofilms (grown in a continuous-culture flow cell for 4 days). XY (central), XZ (bottom) and YZ (left) plans of each image is shown here. A; PDO300, B; PDO300Δ*alg8*, C; PDO300Δ*pslA*, D; PDO300Δ*pslA*Δ*alg8*, E; PDO300Δ*pelF*Δ*alg8*, F; PDO300Δ*pslA*Δ*pelF*, G; PDO300Δ*pslA*Δ*pelF*Δ*alg8*

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CHAPTER IV

Temporal-spatial regulation of exopolysaccharides in mucoid and non mucoid *Pseudomonas aeruginosa* biofilms

Aamir Ghafoor, Stacey Holbrook, Bernd H.A. Rehm

Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

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ABSTRACT

Pseudomonas aeruginosa forms biofilm at biotic and abiotic surfaces. These biofilms are resistant to antibiotic and disinfectant treatments. Biofilm matrix plays an important role in architecture and function of the biofilm. Three exopolysaccharides, Psl, Pel and alginate are important components of the biofilm matrix. Biosynthesis of these three exopolysaccharides is controlled by three operons which are under tight control of their respective promoters. Here, we have shown the temporal and spatial regulation of the *psl*, the *pel* and the *algD* operons. Promoters of the respective operons were fused with *gfp* gene and expression of the GFP was studied by observing biofilms through confocal laser scanning microscopy. In wild type non mucoid PAO1, the results showed that *psl* operon was expressed throughout the PAO1biofilm structure at all timepoints and nearly 90% of the biofilm was populated by the cells showing the *psl* operon expression. The cells showing expression of the *algD* operon were present only in the base of PAO1 biofilms. The biofilms formed by the mucoid strain PDO300 (an isogenic *mucA* mutant of PAO1) were populated by more than 90% cells which were expressing the *algD* operon. However, the cells expressing the *psl* operon were very few and present near the base of the PDO300 biofilms. Expression of the *pel* operon showed gradual increase with the age of the biofilm and was mainly observed in the cells near the attachment surface especially in the base of the stalk of the biofilms formed by both PAO1 and PDO300.

INTRODUCTION

Pseudomonas aeruginosa is a gram negative, rod shaped bacterium, commonly found in aquatic environments. A plethora of virulence factors produced by *P. aeruginosa* help the bacterium to cause infections in plants, worms, insects and mammals (1-3). In humans with cystic fibrosis disorder, this opportunistic pathogen causes chronic infection of the lungs. Inability of the lungs to clear the infectious agent ultimately leads to death of the patient. The persistent nature of the infection is attributed to the ability of the bacterium to grow as biofilm (4). *P. aeruginosa* cells produce and recruit the extracellular polymeric substances (EPS) which form biofilm matrix. The matrix encases the bacterial cells and protects them from desiccation, antibacterial agents, oxidizing agents and host defensive processes (5-7).

P. aeruginosa can be grown in a flow cell system to study the development of biofilm and to observe its structures (8). Previous studies have demonstrated that *P. aeruginosa* forms mushroom-like biofilms which comprises of a base, a stalk and a cap. Formation of the typical mushroom-like structure is dependent on the exopolysaccharides produced by *P. aeruginosa* (9). Three exopolysaccharides, alginate, Psl and Pel, have been shown to play essential roles in architecture and development of 3D mushroom like structure of the biofilm (10, 11). It has been demonstrated that cap formation of the mushroom-like biofilm is dependent on the presence of Psl (10, 12). The quantity of alginate produced in biofilm influences the shape of the mushroom-like biofilm (13). The absence of Pel affects the maintenance of PDO 300 biofilms (10). These results suggest that biosynthesis of the aforementioned exopolysaccharides is regulated spatially and temporally to architecture the structure of the biofilm.

The regulation of exopolysaccharides biosynthesis in *P. aeruginosa* is tightly controlled at transcriptional and post-transcriptional levels. At the transcriptional level alginate biosynthesis is under tight control of the *algD* promoter (14). The initiation of transcription at the *algD* promoter requires an association of RNA polymerase and the alternative sigma factor, AlgU (σ22) (14).

There are multiple pathways that regulate *pel* and *psl* operons expression at transcription and post-transcriptional levels. An increased intracellular concentration of c-di-GMP positively regulates *pel* and *psl* expression. FleQ has also been shown to be involved in the regulation of *pel* and *psl* expression. FleQ represses the expression of

the *pel* operon by binding to the *pel* promoter in the absences of c-di-GMP and an increase in intracellular levels of c-di-GMP relieves the repression (15). The expression of *psl* operon is positively regulated by stationary-phase σ-factor, RpoS (16) whereas the expression of both *pel* and *psl* operons is positively regulated by quorum sensing (17). The regulation of the *psl* and the *pel* operons expression has also been shown at post transcriptional level. PelD contains c-di-GMP binding domain and it is suggested that PelD regulates Pel biosynthesis at post-transcriptional level (18). A messenger-RNA binding protein RsmA negatively regulates the *psl* operon expression via blocking translation of *psl* mRNA (16). The involvement of various factors in the regulation of the *psl* and the *pel* operons suggested a complex control of their expression.

These studies have elucidated the regulation of the exopolysaccharide biosynthesis but regarding temporal-spatial regulation of the expression of their operons in the cells inside a biofilm is yet to be determined. Previously Overhage and colleagues (19) showed that in *P. aeruginosa* PAO1 *psl* was constitutively expressed during planktonic growth and repressed after attachment, but during the later stages of biofilm development *psl* operon was expressed again and expression was localized in the centres of microcolonies. In this study biofilms were grown in Pseudomonas Isolation media and observed using confocal laser scanning microscopy.

In this study temporal-spatial regulation of *pel*, *psl* and *algD* operons are observed both in PAO1 and alginate over-producing strain PDO300. Results indicated that *psl* operon expression is more in PAO1 biofilms when compared to that of PDO300. Expression *of* the *pel* operon was seen mostly confined to the biofilm base. The *algD* operon is expressed in most parts of PDO300 biofilms. However, a low expression of alginate was also observed in PAO1 biofilm.

MATERIALS AND METHODS

i) Bacterial strains and growth conditions

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S1. All the strains of *Escherichia coli* used in this study were grown in Luria-Bertani (LB) medium purchased from Neogen (MI, USA) at 37°C. When needed, ampicillin from Sigma-Aldrich (Auckland, NZ) and gentamicin from Sigma-Aldrich (Auckland, NZ) were added to the media at the concentration of 75μg/mL and 10μg/mL, respectively. *P. aeruginosa* PAO1 and PDO300 (20) were grown in LB or Mineral Salt medium (MSM) (21) at 37°C and when required, antibiotics were added at the following concentrations: gentamicin, 100-300 μg/mL and carbenicillin, 300 μg/mL.

ii) Isolation, analysis, and manipulation of DNA

General cloning procedures were performed as described previously (22). DNA primers, deoxynucleoside triphosphate, *Taq*, and Platinum *Pfx* polymerases were purchased from Life Technologies (Auckland, NZ). DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using an ABI310 automatic sequencer.

iii) Construction of *algD* and *pel* promote-probe vectors

The promoter region, located at -854 to 1 bp upstream of the *algD* open reading frame, was amplified using the primers Palg-Kpn-Fw and Palg-Bam-Rv. The amplified region was intermediately cloned into PGEM-T-Easy and sequence was confirmed. The product was hydrolyzed ligated into the *Kpn*1and *Bam*H1sites of the plasmid pPROBE-gfp[AAV]'::ΩGm (23). Similarly the promoter region, located at −1000 to 1 bp upstream of the *pelA* open reading frame, was amplified using the primers Ppel-EcR-Fw and Ppel-Ba-Rev. After the confirmation of the sequence, the product was ligated into the *EcoR*1 and *BamH*1 sites of the plasmid pPROBE-gfp[AAV]'::ΩGm (23). The plasmid pPROBE-gfp[AAV]'::ΩGm encodes for a labile GFP which has a shorter half life (30 minutes) and which was used for temporal resolution of the concerned gene expression. These plasmids were then transferred into the *P. aeruginosa* strains of interest.

iv) Continuous-culture flow cell biofilms

For biofilm analysis, each mutant was grown in continuous-culture flow cells for 4 days at 37°C as previously described (10, 24). The flow cells with three channels were used. Each of the channels had dimensions of 4 mm by 40 mm by 1.5 mm. Each channel was filled with MSM medium and 0.5 mL overnight culture of the respective mutant, containing approximately 2×10^9 cells per mL was inoculated into the respective channel of the flow-cell. To allow the attachment of cells flow-cells were incubated without flow for 4 h at 37°C. The MSM medium was then allowed to flow at a mean flow of 0.3 mL/min. The flow cells were then incubated at 37°C for 24-96 h according to the requirement of the experiment. To monitor the temporal-spatial regulation of the *pel*, the *psl* and the *algD* operons, GFP fluorescence was observed in *P. aeruginosa* biofilms (harbouring the respective promoter-probe vectors) using confocal laser scanning microscopy (CLSM) (Leica SP5 DM6000B). To observe the cells which were not expressing GFP, biofilm was stained using DNA stain SYTO-64 (Molecular Probes, Inc., Eugene, OR, USA). Images were captured with 40x oil-immersion objective lens of CLSM. These images were analyzed using IMARIS software (Bitplane, inc).

Statistical Analysis

Results were statistically analysed and mean values and standard deviations were calculated using Microsoft excel.

RESULTS

i) Temporal-spatial regulation of the *pel* operon

In 24 to 96 h-old *P. aeruginsa* PDO300 biofilm the *pel* operon was expressed near the base of biofilm (Figure 1A&1B). In 24-h-old PDO300 biofilms expression of the *pel* operon was seen in a few cells spread around the attachment surface. After 48 h the expression started concentrating in the stalks of the biofilm (Figure 1A). After 72 h, the *pel* operon expression increased in localized regions at the base of biofilm stalks. After 96 h of biofilm growth, the *pel* operon expression was seen in most parts of biofilm base and localized *pel* expression can also be seen at the base of biofilm stalks (Figure 1B). Comparison of the height of biofilms and height of the *pel* operon expressing-regions indicated that the *pel* operon-expressing cells are localized in the base of biofilm after 24 h when a multilayer base was developed and 54.04% ± 1.93% of the biofilm height was expressing the *pel* operon. At 48 h, the biofilm height had doubled and expression of the *pel* operon was restricted to the 39.22% ± 3.24% of this. After 72 and 96 h of growth, expression of the *pel* operon was confined to basal region at the heights of 17.06% ± 3.24% and 23.42% ± 0.85% of the overall biofilm height, respectively (Table 2).

In PAO1, the *pel* operon was expressed mostly in the cells which were attached to the surface. The expression at 24 and 48 h was mostly in the layer of the cells closest to the surface and was localized to biofilm stalks (1C). An increase of operon expression was observed after 72 h and again after 96 h. Some expression in the periphery of the biofilm can also been seen after 72 h (ID). Comparison of the height of *pel* operon-expressing-regions with the height of biofilms showed that *pel* operon-expressing cells in PAO1 biofilm increased at the late stages of biofilm development. After 48 h post-inoculation, structured biofilms can be seen and *pel* operon expression was seen only in 19. 82 ± 6 .11% of the biofilm height (starting from the base) but after 96 h it increased to 38% ± 1.59% of biofilm height (Table 2).

Table 1. A comparison of heights of the *psl, pel* and *algD* expressing-regions with the heights of the biofilms of *P. aeruginosa*

Strains & operons	Time points (H)	Height of biofilm (μm) ± St. dev	Height the region-expressing GFP (μm) ± St. dev	Percentage height of the region-expressing GFP) ± St. dev
PDO300				
pel	24	41.54 ± 0.93	22.45 ± 0.80	54.04 ± 1.94
	48	101.58 ± 12.98	39.84 ± 3.29	39.22 ±3.24
	72	127.19 ± 13.78	21.71 ± 4.12	17.07 ±3.24
	96	139.61 ± 1.52	32.70 ± 1.18	23.43 ±0.85
psl	24	29.86 ± 1.46	10.11 ± 0.99	33.85 ±3.33
	48	129.67 ± 3.80	22.46 ± 2.55	17.32 ±1.97
	72	153.79 ± 5.18	2.34 ± 0.50	1.52 ±0.32
	96	123.57 ±5. 83	35.47 ± 2.55	28.71 ±2.06
algD	24	28.86 ± 1.46	26.44 ± 3.72	92.26 ±12.98
	48	119.67 ± 3.80	121.46 ± 1.49	93.15 ±1.25
	72	155.79 ± 5.18	140.01 ± 1.64	89.87 ±1.05
	96	160.24 ± 9.13	135.57 ± 2.45	84.61 ±1.53
PAO1				
pel	24	23.07 ± 2.77	8.40 ± 1.41	36.41 ± 6.11
	48	50.21 ± 6.73	9.96 ± 1.16	19.83 ± 2.31
	72	79.17 ± 1.94	11.81 ± 1.49	14.92 ± 1.88
	96	103.80 ± 2.54	39.63 ± 1.65	38.18 ± 1.59
psl	24	21.23 ± 0.81	20.10 ± 0.91	94.65 ± 4.29
	48	48.26 ± 4.97	37.47 ± 3.50	77.64 ± 7.24
	72	84.73 ± 7.88	65.31 ± 1.31	77.08 ± 1.54
	96	92.23 ± 4.36	59.44 ± 1.88	64.45 ± 2.04
algD	24	26.70 ± 0.49	6.68 ± 0.55	25.02 ± 2.04
	48	76.73 ± 4.50	9.32 ± 0.99	12.15 ± 1.28
	72	81.71 ± 2.38	10.40 ± 0.93	12.73 ± 1.13
	96	99.91 ± 15.05	42.65 ± 9.41	42.69 ± 9.41

ii) Temporal-spatial regulation of the *psl* operon

In 24-h-old PDO300 biofilms the expression of the *psl* operon was seen in cells spread around the attachment surface. After 48 h, the *psl* operon expression started localizing at the base of stalks of the biofilm (1A). Interestingly, the expression of the *psl* operon was in those cells located at the periphery of the stalks. After 24 h post-inoculation, the *psl* operon expression was seen in those cells which were attached to the surface and in only 23.85% ± 3.32% of the biofilm height (Table 2). After 48 h, when structured biofilms were formed, expression of the *psl* operon was confined to 17.32% ± 1.97% of the biofilm height. No expression could be observed in the biofilm after 72 h post-inoculation only some weak signals were seen only in 1.5% ± 0.32% height of the biofilm close to the attachment surface (Figure 1B). After 96 h *psl* operon expression appeared again and was seen in 28.71% ± 2.06% of the height of the biofilm (Table 2).

In contrast, in PAO1 biofilms, expression of the *psl* operon was detected in most of the cells in the biofilm. After 24 h the *psl* operon expression was seen in 94.64% ± 4.28% of the biofilm height. After 48, 72 and 96 h *psl* operon expression was observed in 77.63 % ± 7.24%, 77.07% ± 1.54% and 64.44% ± 2.04% of biofilm heights, respectively.

iii) Temporal-spatial regulation of the *algD* operon

In PDO300 biofilms, alginate operon expression was detected throughout the biofilm at all time points (1A & 1B). After 24, 48, 72 and 96 h post-inoculation, 92.26% ± 12.98%, 93.15% ± 1.25%, 89.87% ± 1.05% and 84.61% ± 1.53% height of the biofilm showed *algD* operon expression (Table 2).

In PAO1 biofilms, expression of the algD operon was seen in the base of the biofilm mostly in the cells attaching to the surface. Interestingly, expression increased at later stages of biofilm development and, after 96 h most of the cells in the biofilm showed expression of the *algD* operon but with much lower intensity (Figure 1C &1D). After 24 h post-inoculation expression of the *algD* operon was detected in 25.02% ± 2.04% height of the biofilm, while the expression was reduced to 12.15% ± 1.28 %, and 12.73% ± 1.13% of the height of the biofilm after 48 and 72 h, respectively. An increase in expression of the *algD* operon was observed after 96 h 42.68% ± 12.46% of biofilm height was populated with cells showing the expression of the *algD* operon.

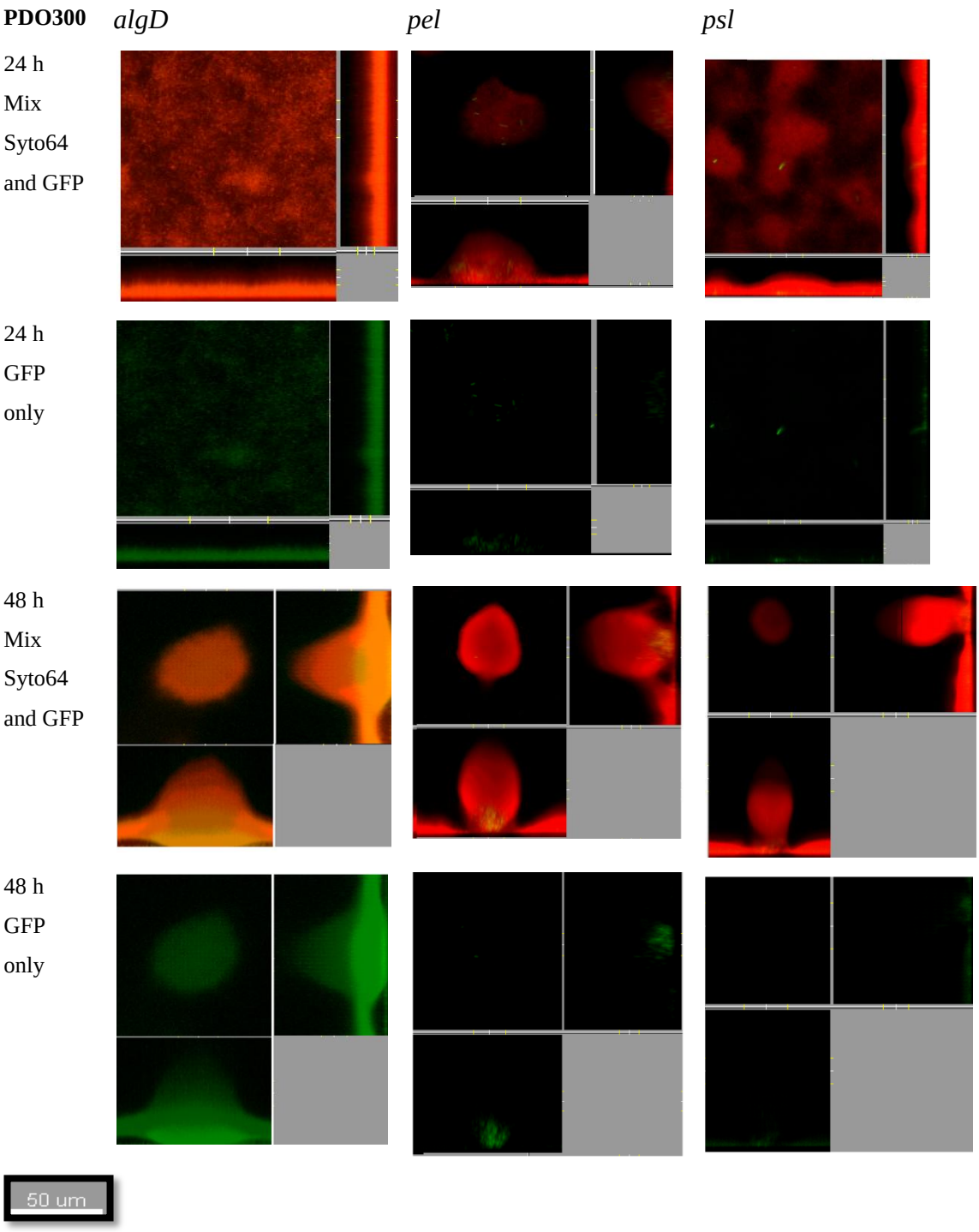

Figure 1A. Expression of the *algD*, the *pel* and the *psl* operons in *P. aeruginosa* PDO 300, 24 and 48 h-old biofilms. XY (central), XZ (bottom) and YZ (left) plans of each image are shown. The red colour of the biofilms is due to SYTO-64 cell-permeable nucleic acid stain. The green fluorescence indicates expression of GFP inside the biofilms. A 50µm bar is provided at the right corner of the figure.

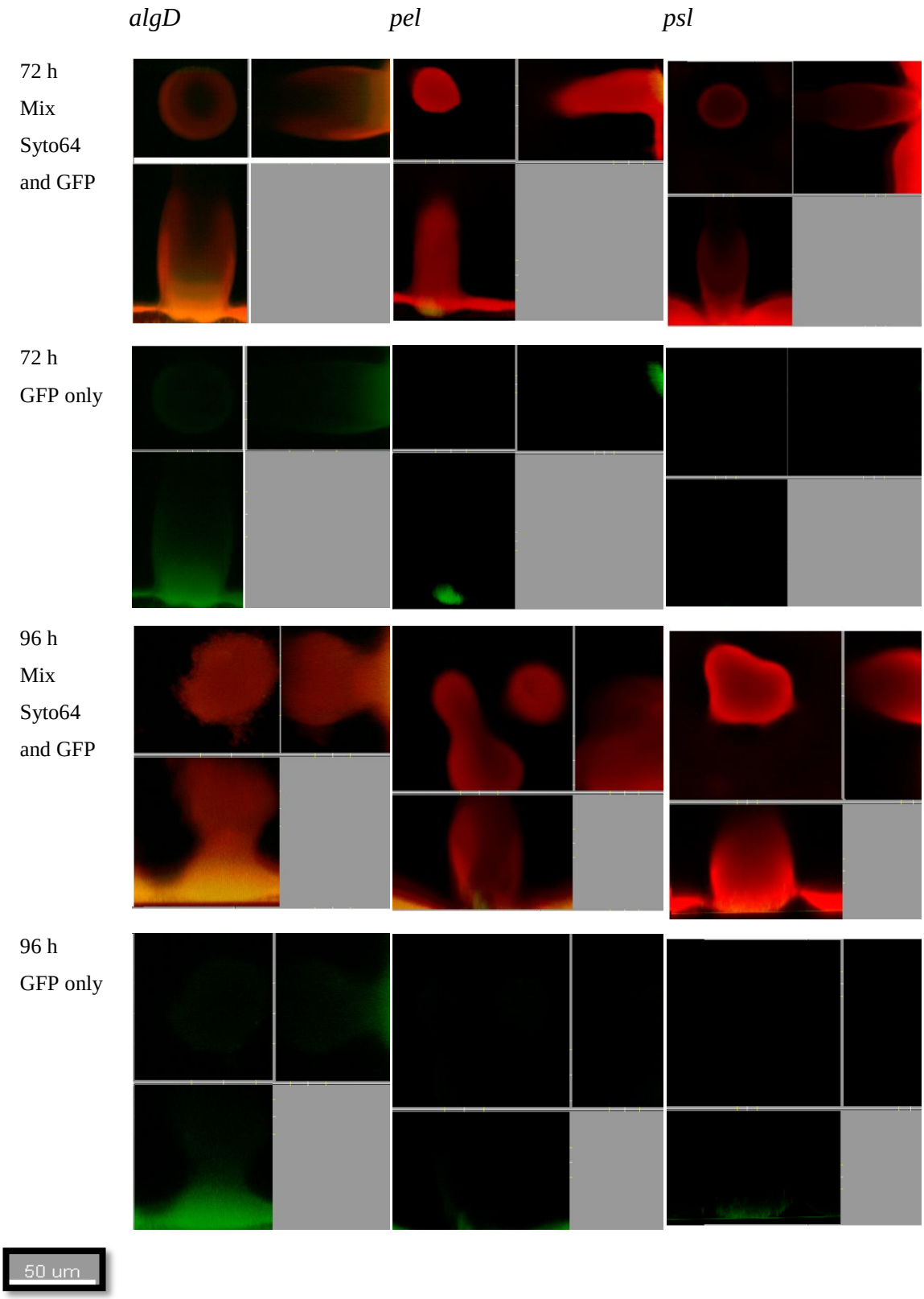

Figure 1B. Expression of the *algD*, *pel* and *psl* operons in *P. aeruginosa* PDO300, 72 and 96 h-old biofilms. XY (central), XZ (bottom) and YZ (left) plans of each image are shown. The red colour of the biofilms is due to SYTO-64 cell-permeable nucleic acid stain. The green fluorescence indicates expression of GFP inside the biofilms. A 50µm bar is provided at the right corner of the figure.

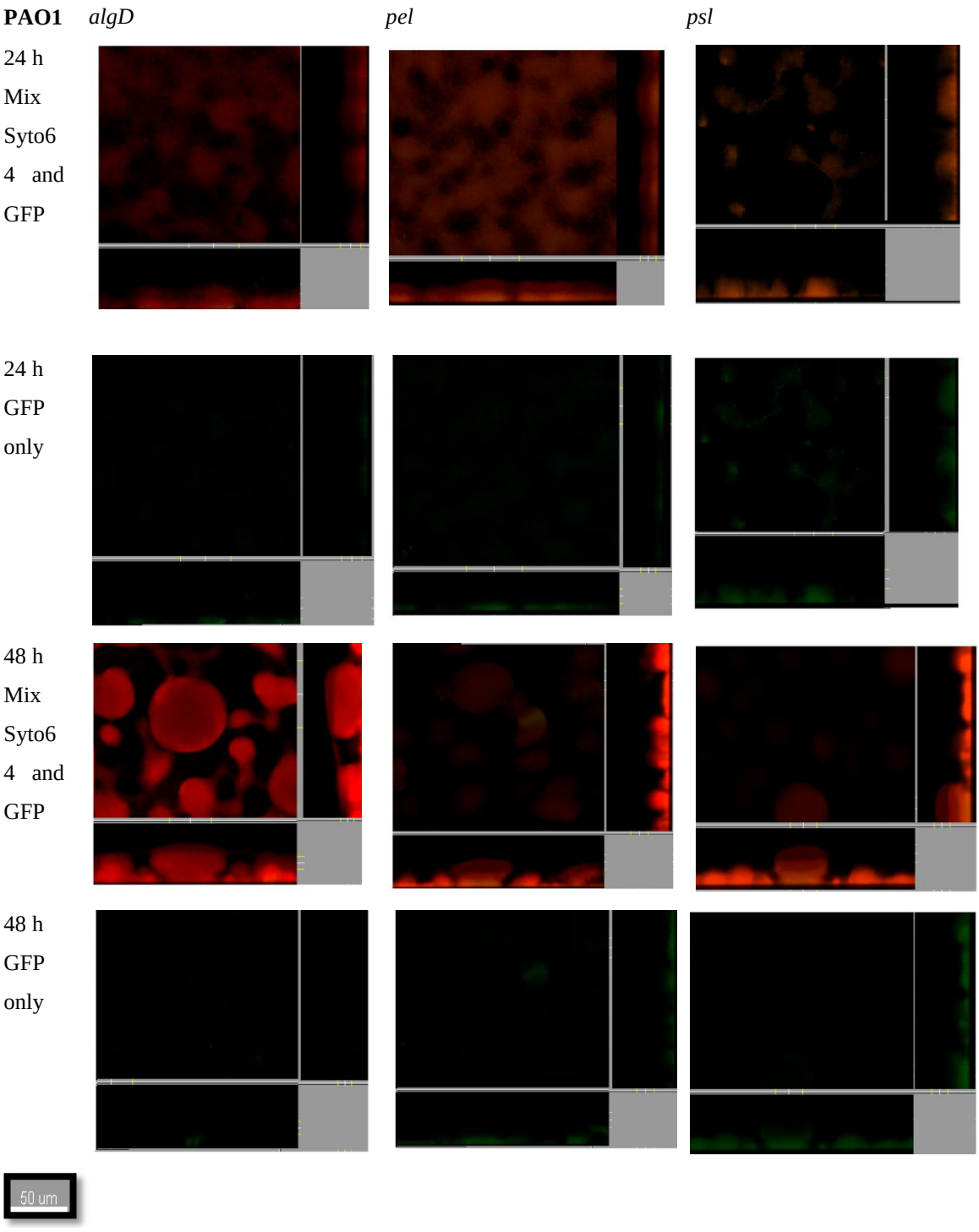

Figure 1C. Expression of the *algD*, *pel* and *psl* operons in *P. aeruginosa* PAO1, 24 and 48 h-old biofilms. XY (central), XZ (bottom) and YZ (left) plans of each image are shown. The red colour of the biofilms is due to SYTO-64 cell-permeable nucleic acid stain. The green fluorescence indicates expression of GFP inside the biofilms. A 50µm bar is provided at the right corner of the figure.

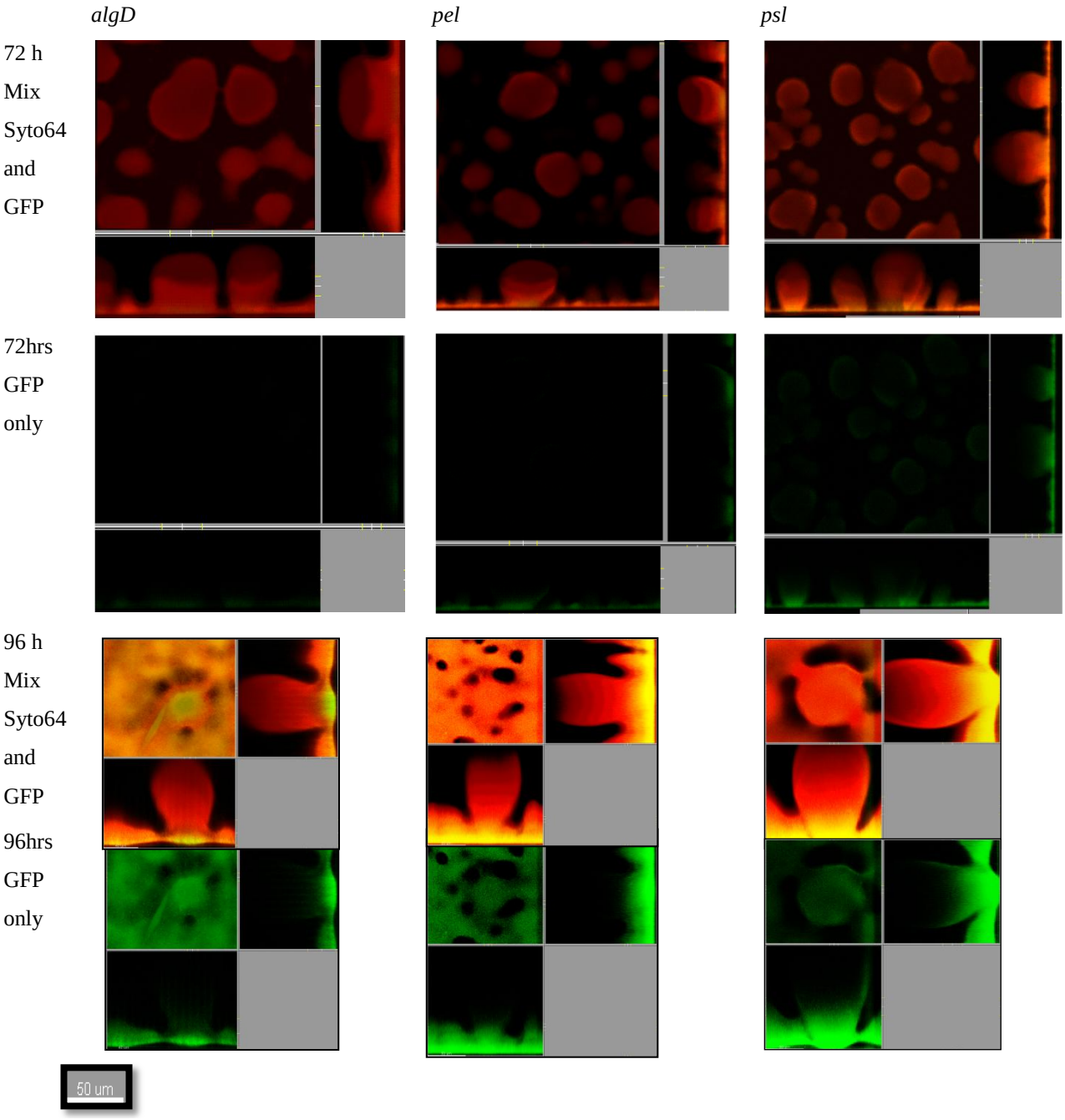

Figure 1D. Expression of the *algD*, *pel* and *psl* operons in *P. aeruginosa* PAO1, 72 and 96 h-old biofilms. XY (central), XZ (bottom) and YZ (left) plans of each image are shown. The red colour of the biofilms is due to SYTO-64 cell-permeable nucleic acid stain. The green fluorescence indicates expression of GFP inside the biofilms. A 50µm bar is provided at the right corner of the figure.

DISCUSSION

In this study *psl*, *pel* and *alg* promoters were fused with GFP encoding genes, respectively, in order to observe the temporal and spatial regulation of respective exopolysaccharides biosynthesis genes in *P. aeruginosa* biofilms. To observe the locaalization of GFP expression, the biofilm was stained with SYTO-64. This is a, red fluorescent, cell-permeant nucleic acid stain which binds to the negatively charged DNA. Bacterial cells when stained with SYTO-64 are red when excited with the light of wavelength 560nm, i.e. an emission spectrum ranging from wavelength 640-700 nm can be observed. The labile GFP used in this study has a shorter half life as compared to stable GFP (23). This allows to observe the expression of GFP at differnet timepoints because if expression of the *gfp* gene is 'switched off', previously accumulated GFP is degraded by the cells.

In the present study, we showed that the *pel* operon expression is mostly confined to the base of *P. aeruginosa* biofilm and its expression increased with the age of the biofilm (Figure 1). Previous studies have shown that the Pel exopolysaccharide plays its role in later stages of the biofilm development (25, 26). A recent study demonstrated that *pelA* transcription was many times higher in biofilm when compared to planktonic cells and *pelF* was expressed only in biofilm cells (27). These results have suggested that the *pel* operon is not expressed by planktonic cells and only biofilm-forming cells showed its expression. In the present study a relatively high expression of the *pel* operon was observed only at the base of the biofilm which suggests the role of Pel in cells attachment to the surface. Other studies have also shown that Pel is required for the attachment of cells in Psl-deficient strain PA14, not in initial stages but at later stages. However, Pel has been shown as a redundant exopolysaccharide when Psl is produced in PAO1 biofilm (26). In PAO1 biofilm Pel expression increased on after 72 h. Expression was observed in the peripheral wall of the cap of mushroom-like biofilm but at the same time Psl expression was also observed in the biofilm. This further suggests the redundant role of Pel in PAO1. This increase in the expression of Pel may influence the compactness of the biofilm. Previous study suggested that Pel-deficient PAO1 biofilms were less compact when compared to wild type (10). It has also been shown that in the absence of Pel a structured biofilm can be formed by PAO1but PDO300 biofilm were not able to maintain biofilm (10). In this study results showed

that the *psl* operon showed reduced expression in PDO300 and the *pel* operon expression was found concentrated at the base of the stalk (Figure 1A & 1B).

Psl is the most important exopolysaccharide shown for *P. aeruginosa* biofilm development. *P. aeruginosa* cells cannot form the mushroom-like biofilm in the absence of Psl (10). In PAO1 biofilms Psl was shown expressed in all cells but an increased expression was observed in the peripheral wall of the biofilm (Figure 1). Previously, it has been shown that Psl is distributed in the periphery of mushroom-shaped structure (12).

Interestingly, *psl* operon expression in PDO300 was very less when compared to PAO1 (Figure 1) and this is consistent with previous study in which we showed that *psl* operon expression was less in PDO300 as compared to PAO1 when cells were grown on solid media (10). A previous study suggests that the presence of uncontrolled AlgU in PDO300 enhances the production of another transcription factor, AmrZ, which down regulates the transcription of *psl* operon (28). Therefore, Psl biosynthesis is reduced at the transcriptional level.

The alginate exopolysaccharide is produced in excess in mucoid strain PDO300 due to unleashed presence of sigma factor AlgU. In PAO1, AlgU is post-translationally controlled by an anti-sigma factor, MucA (14, 29). In this study, we showed that alginate expression can be seen in PDO300 cells populating biofilm structure. This can be explained by the active AlgU that results in the constitutive expression of the *algD* operon. (Figure 1A & 1B).

Alginate operon expression in PAO1 is very much less but an increased expression was seen after 96 h (Figure 1C&1D). Until now, the production of alginate has been controversial in non-mucoid biofilms. Previously it was demonstrated that PAO1 biofilm architecture was changed in the absence of alginate suggesting that alginate was produced in PAO1 strain (10). In addition, another study, using alginate specific antibodies also showed that a very low amount of alginate was produced in the PAO1 biofilms (30). Results of our study are consistent with these above mentioned studies. In contrast, many microarray-based studies suggested that the *algD* operon is not expressed in non-mucoid *P. aeruginosa* biofilms (31-33).

Temporal-spatial regulation of the exopolysaccharide operons can be explained in relation to availability of oxygen. It has been shown that biofilm height affects the availability of oxygen inside the biofilm and a biofilm thicker than 25 µm showed

decreased oxygen near the base (34). This differential oxygen supply creates microenvironments and may affects the gene expression of the subpopulation of cells in the biofilm [(35) and reviewed in (36)]. Interestingly, in PAO1 *algD* operon expression was seen at the base of the biofilm where oxygen supply could be minimum suggesting that decreased oxygen availability may increase the expression the *algD* operon. Previous study has shown that alginate biosynthesis increases in the hypoxic environment (37). That study also suggests that increased amount of alginate decreases oxygen diffusion in the cells encased in alginate (37). Similarly, increased expression of the *psl* operon was shown in cells on the surface of the biofilm (Figure 1C & 1D) where oxygen supply is the maximum. This indicates that *psl* expression is increased in the presence of oxygen. Decreased expression of the *psl* operon in alginate overproducing strains could be due to decreased oxygen supply in the biofilm because alginate does not allow oxygen to diffuse into the biofilm (37). Expression of the *pel* operon was also seen close to the base of the biofilm which indicate its relation with hypoxic conditions.

Overall, this study demonstrates that Psl is the main exopolysaccharide produced in the biofilms formed by non-mucoid strain, PAO1. Whereas, the mucoid strain, PDO300 increases the expression of the *algD* operon during biofilm formation and this increased alginate production negatively regulate expression of the *psl* operon. When *algD* operon is constitutively expressed, the *psl* operon expression is significantly reduced. This study also showed the importance of Pel in the stalk of PDO300 especially in the absence of Psl.

SUPPLEMENTARY MATERIAL

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

Strains, plasmids, or oligonucleotide	Description or sequence (5´-3´)	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototrophic wild-type non mucoid strain	(38)
PDO300	*mucA22* isogenic mutant derived from PAO1	(20)
E.coli.		
TOP10	*F-mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*	Invitrogen
Plasmids		
pProbeAT’	Broad-host-range *gfp* (stable form) fusion vector	(23)
pProbeAT_::P$_{psl}$	pProbeAT containing EcoRI/BamHI PCR fragment of *psl* promoter	(19)
pProbeAT_::P$_{pel}$	pProbeAT containing EcoRI/BamHI PCR fragment of *pel* promoter	This study
pProbeAT_::P$_{algD}$	pProbeAT containing EcoRI/BamHI PCR fragment of *algD* promoter	This study
pProbe_-gfp[AAV]’	Broad-host-range *gfp* (unstable form) fusion vector	(23)
pProbe-gfp[AAV]’::ΩGm-P$_{psl}$	pProbe_-gfp[AAV]::P*psl* containing a gentamicin restistance gene cassette in the opposite direction to that of the *gfp* gene	(19)
pProbe-gfp[AAV]’::ΩGm-P$_{pel}$	pProbe_-gfp[AAV]::P*pel* containing	This Study

	a gentamicin restistance gene cassette in the opposite direction to that of the *gfp* gene	
pProbe-gfp[AAV]'::ΩGm-P$_{algD}$	pProbe_-gfp[AAV]::P*algD* containing a gentamicin resistance gene cassette in the opposite direction to that of the *gfp* gene	This Study
Oligonucleotides		
Ppel- EcRfor	AAAGAATTCGCGCTCGCACGC CGTTACGGCAC	
Ppel- BaRev	AAAGGATCCGCCCAGCCTACG CGGCAGGGTCG	

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CHAPTER V

The role of PelF in Pel polysaccharide biosynthesis in *Pseudomonas aeruginosa*

Aamir Ghafoor, Zoe Jordens, Bernd H.A. Rehm

Institute of Molecular Biosciences, Massey University, Private Bag 11222, Palmerston North, New Zealand

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ABSTRACT

Pseudomonas aeruginosa produces three exopolysaccharides, Psl, Pel and alginate, which play vital roles in biofilm formation. Pel is a glucose-rich, cellulose-like exopolysaccharide. The essential Pel biosynthesis proteins are encoded by seven genes, *pelA-G*. Bioinformatics analysis suggested that PelF is a cytosolic glycosyltransferase. Here, experimental evidence was provided to support this PelF function. A UDP-glucose dehydrogenase based assay was developed to quantify UDP-glucose. UDP-glucose was proposed as substrate for PelF. The isogenic *pelF* deletion mutant accumulated 1.8 times more UDP-glucose in its cytosol than the wild type. This suggested that PelF, which was found localized to the cytosol, uses UDP-glucose as substrate. Additionally *in vitro* experiments confirmed that PelF uses UDP-glucose as substrate. To analyze the functional roles of conserved residues in PelF, site-directed mutagenesis was performed. The presence of the EX_7E motif is characteristic for various glycosyltransferase families and in PelF E405/E413 are the conserved residues in this motif. Replacement of E405 with A resulted in reduction of PelF activity to 30.35 ± 3.15% of wild type whereas replacement of second E413 with A did not show a significant change in the activity of PelF. Moreover replacement of both E residues did not result in a loss of PelF function, but replacement of conserved R325 or K330 with A, respectively resulted in complete loss of PelF activity. Overall our data showed that PelF is a soluble glycosyltransferase which uses UDP-glucose as substrate towards Pel synthesis and that conserved residues R325 and K330 are important for the activity of PelF.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen responsible for chronic pulmonary infections in cystic fibrosis patients. It causes persisting infections due to its ability to form biofilms (1). Inside the biofilm matrix, bacterial cells are protected from adverse effects of antibiotics and the host immune response (2). The biofilm matrix is mainly composed of extracellular DNA (eDNA), proteins and exopolysaccharides (EPS). Three important exopolysaccharides that are synthesized and secreted by *P. aeruginosa* are alginate, Psl and Pel (3). Pel is biosynthesis machinery is encoded by the *pel* operon which comprises of seven genes (*pelA*-*G*).

The role of the proteins encoded by the *pel* operon had only been determined for PelC and PelD. PelC is an outer membrane lipoprotein presumably involved in transportation of Pel to the bacterial cell surface (4) whereas, PelD is a bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP) binding protein that is involved in post-translational regulation of Pel production (5). The role of PelA, PelB, PelE, PelF and PelG has not yet been shown experimentally. However, on the basis of sequence homology it has been predicted that PelG could be a member of polysaccharide transporters (PST) family; PelD and PelE, the proposed inner membrane proteins, are presumably involved in the transferring of Pel across the cytoplasmic membrane; PelA displays weak sequence homology with glycosylhydrolase enzymes (6). PelB has been proposed as a multidomain protein containing a periplasmic and an outer membrane domain. The C-terminal domain is proposed to contain a β-sheet structure and suggested to be an outer membrane protein, like AlgE (7). This domain might functions as a porin involved in polysaccharide secretion. Whereas, homology modelling suggested that the N-terminal periplasmic domain shows similarity with anaphase-promoting complex/cyclosome subunit Cdc 16/Cut9 (PDB ID:2XPI). This domain might be involved in protein-protein interaction due to the presence of tetratricopeptide-like repeats (TPR) (8). Bioinformatics analysis of PelF suggested that it is a glycosyltransferase.

Biosynthesis of polymers in bacteria is under extensive research. A comprehensive analysis of polymers biosynthesis in bacterial species has been previously reviewed (9). Glycosyltransferases are required for initiation or elongation of carbohydrate chains during polysaccharide biosynthesis. These enzymes transfer an activated mono- or oligosaccharide residue to an existing acceptor molecule forming

glycosidic bonds. Glycosyltransferases use a nucleotide phosphosugar (Leloir-type) or an oligosaccharide (Non- Leloir-type) as the glycosyl donor; and monosaccharides, oligosaccharides, polypeptides, nucleic acids and lipids as acceptors to catalyze the formation of a glycosidic bond. The reaction can result in inversion or retention of the anomeric configuration of the donor sugar in the product and as such can be referred to as inverting or retaining glycosyltransferases respectively (1). The Carbohydrate Active Enzyme (**CAZy)** database (www.cazy.org) has divided all glycosyltransferases into 94 families (till July 2012) using the classification described by Campbell et al. (10) and by Coutinho et al. (11). Despite great primary structure diversity, the tertiary structure of most glycosyltransferases is conserved. All structures of nucleotide-sugar-dependent glycosyltransferases solved to date have shown only two general folds, termed GT-A and GT-B (11-14). Bioinformatics analysis has suggested the presence of a third fold, GT-C (15). The GT-A and GT-B are Rossman-like folds which consist of two β/α/β domains. In the GT-A fold, both domains are closely associated forming a compact globular structure displaying distinct nucleotide- and acceptor-binding sites (14). A DXD motif is commonly found in GT-A enzymes in which the glutamic acids' carboxylate groups coordinate a divalent cation (Mg^{2+} and Mn^{2+}) and/or a ribose (10, 11). Although the DXD motif is considered to be a signature of GT-A glycosyltransferases, it has been found that this motif is not absolutely conserved. (16). Alternatively, the DXD motif is present in many proteins which are not glycosyltransferases. The GT-B fold consist of two less-tightly associated domains facing each other in such a way that a cleft containing the active site is formed. In contrast to the GT-A fold, the GT-B fold lacks any DXD motif and as such, work by a metal ion-independent mechanism (17). Most of the CAZy GT4 family glycosyltransferases contain a conserved EX_7E motif at their C-terminus (11). These two conserved glutamic acid residues are suggested to be involved in catalytic activity but their role has not been confirmed, yet (11).

PelF belongs to glycosyltransferase family-4 (GT-4) of the CAZy data base. Members of the GT-4 family are retaining glycosyltransferases that display a GT-B fold. In this study, the functional role of PelF was investigated. The *in vivo* and *in vitro* activity of PelF was studied with respect to its role in biosynthesis of Pel and the formation of pellicle at the air-liquid interface. In this study, the key amino acid residues essential for function of PelF have been identified.

MATERIALS AND METHODS

i) Bacterial strains and growth conditions

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table S1. The strains of *Escherichia coli* were grown in Luria-Bertani (LB) medium purchased from Neogen (MI, USA) at 37°C. When needed, ampicillin from Sigma-Aldrich (Auckland, NZ) and gentamicin from Sigma-Aldrich (Auckland, NZ) were added to the media at the concentration of 75μg/mL and 10μg/mL, respectively. *P. aeruginosa* PAO1 were grown in LB or Pseudomonas Isolation broth (20 g of peptone, 10 g of K_2SO_4, 1.4 g $MgCl_2.6H_2O$, 25 mg of triclosan, and 20 mL of glycerol per liter) at 37°C and if required, antibiotics were added at the following concentrations: gentamicin, 100-300 μg/mL and carbenicillin, 300μg/mL.

ii) Construction of plasmid encoding His10 tagged PelF

To construct the His-tagged PelF-encoding plasmid, *pelF* was amplified by PCR with the primers PelF-His-Frw and PelF Rev (Table S1) using plasmid pBBR1MCS-5::pelF (3) as the DNA template. All procedures were performed as described previously (18). Deoxynucleoside triphosphate, *Taq*, and Platinum *Pfx* polymerases were purchased from Life Technologies (Auckland, NZ). The product was inserted into pGEMT-Easy, purchased from Promega (Sydney, Australia), and the resulting plasmid propagated in *E. coli* Top10 and isolated with a High Pure plasmid isolation kit purchased from Roche (Auckland, NZ), according to manufacturer's instruction. The DNA sequence was confirmed by DNA sequencing according to the chain termination method using an ABI310 automatic sequencer. The plasmid was transformed into *E. coli* JM110 to avoid methylation of the *Xba*1 site. pGEMT-easy-His_{10}-PelF purified from *E. coli* JM110 was digested with *Nde*1 and *Xba*1 to obtain the fragment encoding His_{10}-PelF. Plasmid pBBR1-MCS5:: *alg8* (19), was digested with *Nde*1 and *Xba*1 to remove the *alg8* gene and ligated with the Nde1-His_{10}-PelF-Xba1 fragment to obtain plasmid pBBR1-MCS5::His_{10}-PelF encoding His-tagged PelF. A ribosomal binding site was already available upstream of *Nde*1 in the plasmid. All inserts cloned into the multiple cloning site of the vector were under the control of *lac* promoter.

iii) *In vivo* activity of PelF

The plasmid pBBR1-MCS5::His_{10}-PelF was used to transform Δ*pelF* mutants of *P. aeruginosa* for complementation experiments. The *in vivo* activity of PelF was assessed using pellicle formation assays and Congo red binding assays as previously described (3).

iv) Subcellular localization of PelF

P. aeruginosa strains were grown for 12-14 h in LB. This culture was diluted 1:50 in fresh LB broth and then grown for 8 h to obtain an optical cell density at 600 nm of 1.5 to 1.6. The cells were collected by centrifugation at 5000 x *g* for 10 min at 4ºC. Cell sediments were washed twice with saline (150 mM NaCl, pH 7.2) and then the cells resuspended in 50 mM HEPES buffer (pH 7.4). The cell suspension was sonicated at 30% intensity for 10 cycles of 15 s sonication, followed by 10 s of cooling. Cellular debris and unlysed cells were removed by centrifugation at 15,000 x *g* for 20 min at 4ºC, and the supernatant was centrifuged at 100,000 x *g* for 2 h. The supernatant (soluble fraction) was transferred to a clean tube and used for sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The sediments were re-suspended in 800 µL of 50 mM HEPES buffer (pH 7.4) and washed by centrifugation at 100,000 x *g* for 2 h. The washing procedure was repeated twice. This washed pellet was used for SDS-PAGE and immunoblotting.

v) Analysis of proteins

Protein extracts from *P. aeruginosa* containing plasmids pBBR1-MCS5 (negative control) and pBBR1-MCS5::His_{10}-PelF (encoding His_{10}-PelF-) were separated by SDS–PAGE (20). 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 manufacturer's manual (SuperSignal® West HisProbe™, Pierce).

vi) Enrichment of PelF

The His_{10} tagged PelF was enriched from protein extracts using His-Spin Protein Miniprep™ columns (Zymo Research) in HSMP buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10 mM 2-Mercaptoethanol, and 1x Roche EDTA-free complete protease inhibitor). *P. aeruginosa* strains, containing pBBR1-MCS5:His_{10}-PelF, were grown in 500 mL PI broth for 14 h at 37ºC with shaking. Cells were collected by centrifugation at 15,000 x *g* for 2 min, and the cell pellet was washed twice with saline (150 mM NaCl, pH 7.2) and resuspended in 5mL of HSMP buffer containing 10 mM Imidazole. The cell pellet was lysed by sonication as described for subcellular fractionation above. The cell lysate was centrifuged at 15,000 x *g* for 20 min to remove unlysed cells and cell debris and 5 mL supernatant was collected for further treatment. A volume of 250 µL of supernatant was incubated in a His-Spin Protein Miniprep column for 3 min and then centrifuged to remove unbound proteins. This was repeated until the whole 5 mL supernatant had been processed through the column. Finally the column was washed thrice with HSMP buffer containing 50 mM Imidazole and protein was eluted with 100 µL of HSMP buffer containing 300 mM Imidazole. *P. aeruginosa* PAO1 Δ*pslA*Δ*pelF* cells containing plasmid pBBR1-MCS5 were subjected to the same method to obtain PelF-deficient elution fraction. Both His_{10}-PelF-containing and PelF-deficient elution fractions were analysed by SDS-PAGE.

vii) The Quaternary structure analysis by gel filtration chromatography

Elution fractions containing PelF were loaded onto a Superdex S-200 10/300 GL column (GE Healthcare, Piscataway, NJ) pre-equilibrated with 50 mM phosphate (pH 7.6), 150 mM NaCl, and 10 mM β-mercaptoethanol. A flow rate of 0.3 mL/min was used.

viii) PelF and co-purified proteins identification

Proteins that were abundant and with an apparent molecular weight as expected for PelF as well as three other proteins with apparent molecular weights of 88kDa, 35kDa and 19kDa, respectively were analyzed by tryptic peptide fingerprinting using matrix

assisted laser desorption ionization- time of flight- time of flight (MALDI-TOF-TOF) mass spectrometry. The analysis was conducted by the Centre for Protein Research (CPR), Departments of Biochemistry and Microbiology & Immunology Otago University, New Zealand.

ix) Quantification of UDP-glucose in cell lysates

Overnight cultures of Pel-producing and Pel-deficient mutants were diluted 1:50 in fresh PI broth and incubated at 37ºC for 12-14 h until an optical cell density at 600 nm of 2.0 was obtained. Cultures were collected by centrifugation at 5000 x *g* for 10 min at 4ºC and cells were washed twice with saline. Washed cells were diluted in HSMP buffer containing 10 mM Imidazole. Cells were lysed by sonication as described above. The cell lysate was centrifuged at 15,000 x *g* for 20 min at 4ºC to remove cellular debris and unlysed cells. Finally, the supernatant was centrifuged at 100,000 x *g* for 2 h to obtain the soluble fraction. To account for the amount of bacteria in the supernatant of each sample, quantification of UDP-glucose assay was normalized to the amount of total protein in each sample. Total protein concentration in all supernatants (subcellular fraction) was measured using the Bradford protein assay kit (Bio Rad). A volume of 50µL of each soluble fraction was added to the respective well of 96-well flat-bottomed Greiner µClear® plate and the absorbance at 340 nm was recorded. Each well containing a soluble fraction was mixed with 0.01 units of Calbiochem® UDP-glucose dehydrogenase (La Jolla, CA) and 4mM NAD+ (Sigma). The reaction mixtures were incubated at 30ºC for 0, 10, 15, 20, 25, 30, 35, 40, 45 min and the the absorbance at 340 nm was recorded after each incubation period. The quantity of NADH produced in the reaction at each time point was calculated using the absorbance at 340 nm (ϵ_{NADH} = 6220 M^{-1} cm^{-1}). The moles of NADH produced per mL of samples were calculated using Lambert-Beer's law and total UDP-glucose used in the reaction were calculated.

x) PelF activity

Soluble subcellular fractions (30µL) obtained from PelF-deficient mutants were added to 96-well flat-bottomed plates. Half of these wells were mixed with 20 µL of PelF-enriched elution fraction containing 20µg of total protein and the remaining wells were mixed with 20µL PelF-deficient elution fraction as negative control. Mixtures were

incubated at 37ºC for 1 h. After this incubation period, each well was mixed with 0.01 units of UDP-glucose dehydrogenase and 4mM NAD^+, incubated for 0-45 min at 30ºC and the absorbance at 340nm was measured. The concentration of UDP-glucose was calculated using Lambert-Beer's Law as described above.

xi) Purification of Pel oligomers

A single colony of *P. aeruginosa* Δ*alg8* Δ*pslA* (3), an alginate and Psl negative but Pel producing mutant was cultivated in 500 mL of PI media as described above. The Pellicle formed at the air-liquid interface was mechanically removed and washed with MiliQ water. The pellicle was freeze dried and weighed. The dried pellicle was suspended in 200 mL of 1% (w/v) NaOH and then boiled at 100°C for 30 min. After cooling down the digested pellicle material (Pel) was spun down by centrifugation at 7000 x *g* for 20 min. After centrifugation, Pel was dissolved in Cross-Beaver reagent (200mL of 12.1 M HCl mixed with 100g of $ZnCl_2$) and incubated for 15 h at room temperature. After incubation the Pel in Cross-Beaver reagent was slowly mixed with 4 volumes of 95% ethanol. The precipitate formed after gentle shaking was centrifuged at 7000 x *g* for 30 min. The supernatant was discarded and Pel sediment was dissolved in 5 mL phosphate buffer. Purified Pel along with other components was incubated with 15 µg/mL DNase I and 15 µg/mL of RNaseA at 37 °C for 6 h. Then Pronase E was added to a final concentration of 20µg/mL and the solution was incubated at 37 °C for further 18 h. The Pel solutions were dialyzed against 5 L of MilliQ water for 48 h and then freeze dried. The dried Pel was subjected to acetic acid hydrolysis by heating it at 65^{o}C for 2 h and acetic acid was removed by evaporation. The dried material was scratched from the walls of tube and weighed. Pel oligomers were dissolved at the concentration of 500µg/mL in HSMP buffer and used for *in vitro* UDP-glucose dehydrogenase based assay.

xii) *In vitro* UDP-glucose dehydrogenase based assay for PelF activity

Enzyme activity of UDP-glucose dehydrogenase was assessed as previously described (21). To assess the enzymatic activity of PelF, a reaction mixture containing 50 mM HEPES buffer (pH 7.4), 2mM UDP-glucose, 5µg Pel oligomers and 20µg PelF was

made. According to the reaction requirements for known glycosyltransferases co-factors $MgCl_2$ or $MnCl_2$ or $CaCl_2$, each at a concentration of 5mM were added. To assess the role that the contents of different subcellular fractions may play, 5 µL of soluble or inner membrane fractions containing 4mg/mL protein were added to the reaction. To study the role of various sized components in soluble fractions, filtration of soluble fraction was done through filter with 10kDa cut off point. The retentate was dissolved in HSMP buffer containing 300mM imidazole and 5 µL retentate suspension containing 4mg/mL of total protein was added to the reaction. Similarly, filtrate containing 1 mg/mL of total protein was added to reaction. In all cases the total volume was adjusted to 40 µL and set in 96-well flat-bottomed Greiner µClear® plates. Plates were incubated at 37°C for 0, 10, 15, 20, 25, 30, 35, 40, 45 min. All wells were mixed post incubation with 0.01 units of Calbiochem® UDP-glucose dehydrogenase (La Jolla, CA) and 4mM NAD+ (Sigma) and total volume was adjusted to 50 µL. The reaction plates were incubated at 30^oC for 1-10 min. The absorbance at 340nm was measured. The moles of NADH produced per mL of samples were calculated using Lambert-Beer's law and total UDP-glucose used in the reaction were calculated

xiii) Site-directed mutagenesis of *pelF*

The plasmid pBBR1MCS-5::His_{10}-PelF which encodes the His_{10} tagged protein PelF, was used as a template. Site-directed mutations in the coding sequence of *pelF* were created using the Site-directed Ligase Independent Mutagenesis (SLIM) method (22-24). D303A, D362A, E405A, E413A and E405A/E413A mutations were generated using four primers for each mutation. Instead of using all four primers in a single reaction, the primers were split into two pairs to produce two tailed products as described previously (24). Briefly, primers FrwL and RevS for respective mutations were used to produce product A and primers RevL and FrwS were used to produce product B. Both the products A and B were treated with Dpn1 to digest the template plasmids. The two PCR products were mixed in equimolar amounts, and allowed to hybridize by incubating in H buffer (150 mM NaCl, 25 mM Tris, 20 mM EDTA, pH 8.0) at 99°C for 3 min, followed by three cycles of 65°C for 5 min and 30°C for 40 min. Competent *E. coli* TOP 10 cells, prepared by using the $CaCl_2$ procedure (25), were transformed using the hybridized products. Plasmid-containing cells were selected on LB agar plates containing 10µg/mL gentamycin. The plasmids were extracted and

sequenced to confirm mutation. For mutations D301A, D360A, R325A, K330A and K333A fragments Pst1-D301A-Sma1, Pst1-D360A-Sma1 (Genscript), Pst1-R325A-Sma1, Pst1-K330A-Sma1 and Pst1-K333A-Sma1 (IDT) (Table S2), harbouring respective mutations were commercially synthesized by GenScript (Piscataway, NJ) and Integrated DNA Technology through customscience (Auckland, NZ). The plasmid pBBR1MCS-5:His_{10}-PelF was isolated and digested with enzymes *Pst1* and *Sma1*. The 253 bp fragment was replaced with Pst1-D301A-Sma1, Pst1-D360A-Sma1, Pst1-R325A-Sma1, Pst1-K330A-Sma1 or Pst1-K333A-Sma1 fragments to generate D301A, D360A, R325A, K330A and K333A mutations respectively. Competent *E. coli* TOP 10 cells were transformed using plasmids harbouring respective mutations. The mutations were confirmed by sequencing and/or by restriction digest analysis. Confirmed plasmids were transformed into *P. aeruginosa* PAO1 Δ*pslA*Δ*pelF* to observe pellicle formation. Congo red binding assays were performed as previously described and relative percent Congo red binding was measured (3). Congo red results are expressed as a percentage whereby Congo red bound to PelF-producing and PelF-deficient strains was set 100% and 0% respectively.

RESULTS

i) Ability of N terminally His_{10} -tagged PelF to restore pellicle formation in *pelF* deletion mutants.

P. aeruginosa PAO1 Δ*pelF*Δ*pslA* (3) complemented with the plasmid pBBR1-MCS5::His_{10}-PelF produced a pellicle at the air-liquid interface when grown in static culture as shown by a total Congo red staining of 72.5 ± 3.8%. In contrast, the pBBR1-MCS5 harbouring mutant (negative control) was unable to form a pellicle at the air-liquid interface, and a total Congo red staining of only 34 ± 4.1% was obtained.

ii) Subcellular localization of PelF

Envelope and cytosolic fractions of mutants PAO1 Δ*pelF*Δ*pslA* harbouring pBBR1-MCS5::His_{10}-PelF, PAO1 Δ*pelF*Δ*pslA* harbouring pBBR1-MCS5 and PAO1Δ*pslA* were subjected to SDS-PAGE and immunoblotting using anti-His-antibodies. Results showed that the His-tagged PelF was present only in the soluble fraction of PAO1 Δ*pelF*Δ*pslA* harbouring pBBR1-MCS5::His_{10}-PelF. The identified protein showed an apparent molecular weight of 58kDa, which corresponded to the theoretical molecular weight of PelF (Figure 1).

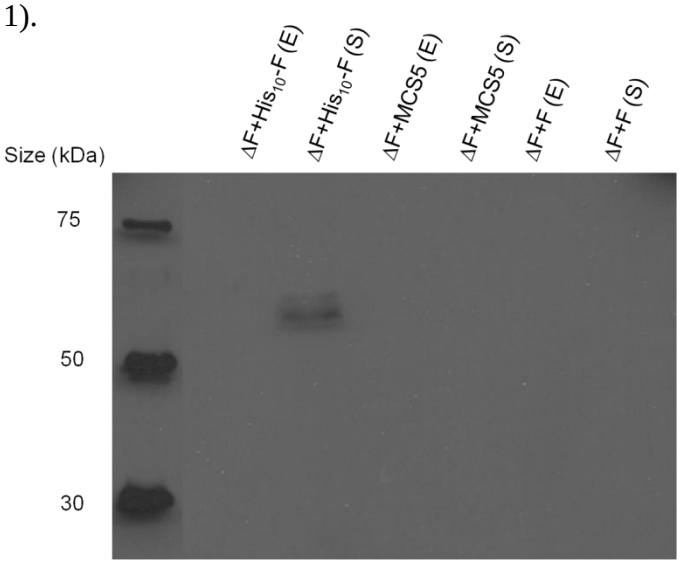


Figure 1. Immunoblot showing the subcellular localization of PelF. Soluble (S) and envelope (E) fractions of *P.aeruginosa* PAO1 *ΔpslAΔpelF* harbouring pBBR1-MCS-5:: His_{10}-PelF, empty vector (pBBR1-MCS-5) and pBBR1-MCS-5::PelF (without His tag) were subjected to SDS-PAGE.

iii) Purification and identification of PelF

The soluble fraction of the His_{10}-PelF-producing strain was subjected to affinity chromatography using Ni-NTA agarose in order to purify His-tagged PelF. His-tagged PelF was partially purified as shown by SDS-PAGE analysis. A distinct protein band exhibiting an apparent molecular weight of 58kDa was obtained (Figure 2). Tryptic peptide fingerprinting analysis in combination with MALDI-TOF/MS enabled identification of this 58kDa protein as His_{10}-PelF (Table S3). The native His10-PelF molecular weight was determined by gel filtration chromatography and resulted in an apparent molecular weight of about 65kDa. The ratio between estimated and actual molecular weight was 1.12, suggesting that the native PelF is present as monomer. Three other proteins with apparent molecular weights 88kDa (A), 34kDa (B) and 19kDa (C), respectively (Figure 2), were co-eluted with PelF.

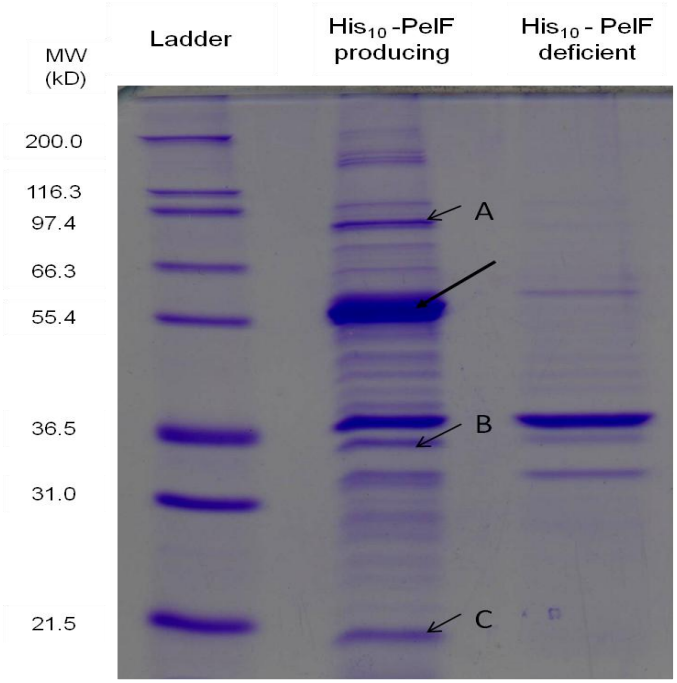


Figure 2. SDS-PAGE analysis of partially purified PelF. Soluble fractions of PelF-producing and PelF-deficient strains were subjected to His-Spin Protein Miniprep™ column and elution fractions were analysed by SDS-PAGE. An arrow (thick) indicates the presence of His_{10}-PelF. Three proteins co-eluting with PelF were identified as: A; ATP-dependent protease (GI: 15595976), B; PA4657 (GI: 15599852) of *P. aeruginosa* and C; Gcn5-related N-acetyltransferase (GI: 239721) (best match to *Serratia marcescens)*.

These proteins were absent in elution fraction when using the PelF-deficient mutant as source. The tryptic peptide fingerprinting analysis in combination with MALDI-TOF/MS showed that the 88kDa protein was an ATP-dependent protease (GI: 15595976) and the 34kDa protein was a hypothetical protein PA4657 (GI: 15599852) of *P. aeruginosa*. The third protein with apparent molecular weight of 19kDa showed the best match with a Gcn5-related N-acetyltransferase (GI: 239721) of *Serratia marcescens*.

iv) Is UDP-glucose the substrate of PelF and a precursor for Pel synthesis?

In order to assess whether the presence or absence of PelF impacts on the intracellular concentration of UDP-glucose, the proposed substrate for PelF, a UDP-glucose dehydrogenase based assay was developed. The UDP-glucose dehydrogenase displayed a typical Michaelis Menton kinetics under these conditions yielding a Km of 77µM and Vmax of 44 moles / minute enabling sensitive detection of UDP-gluocse in the reaction mixture.

The UDP-glucose concentration of the soluble fraction of each the PelF-deficient and PelF-producing strains were analysed using this new method, yielding 0.97 ± 0.06 and 0.533 ± 0.042 µmol/mg protein respectively.

When partially purified His_{10}-PelF- was added to cell lysate of PelF-deficient mutant, the concentration of UDP-glucose was reduced from 1.02 ± 0.06 to 0.66± 0.29 µmol/mg of the total protein when incubated for 30 min. The same elution fraction obtained by purification of a strain lacking His_{10}-PelF did not impact on the total UDP-glucose concentration when added to cell lysate of the PelF-deficient mutant (Figure 3).

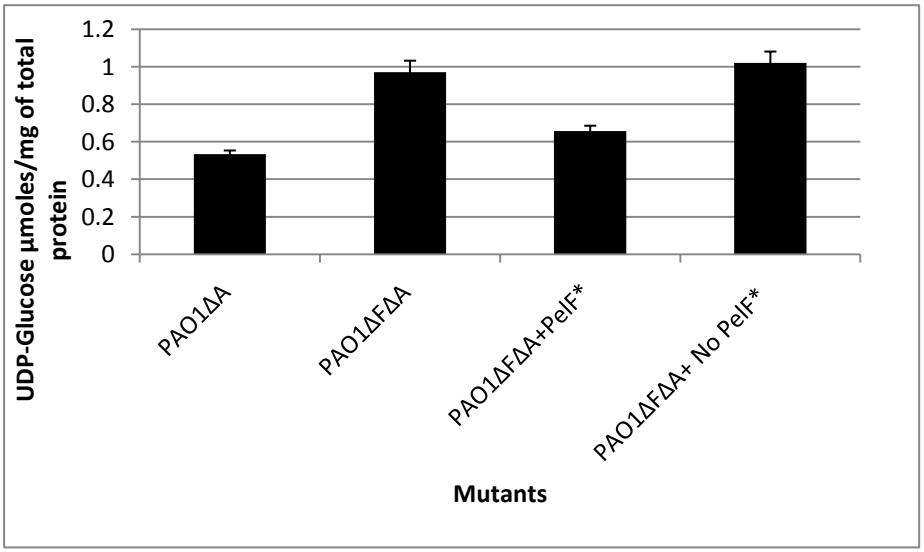


Figure 3. Concentration of UDP-glucose in the soluble fraction of various *P. aeruginosa* PAO1 mutants. PAO1ΔA (PAO1 *ΔpslA*), Psl-deficient/Pel-producing mutant; PAO1ΔFΔA (PAO1 *ΔpelFΔpslA*), Pel-deficient/Psl-deficent mutant; PAO1ΔFΔA (PAO1 *ΔpelFΔpslA*) + PelF, partially purified PelF added to soluble fraction of cell lysate from Pel deficient/Psl-deficent mutant (*PelF was partially purified as described in "MATERIALS AND METHODS"); PAO1ΔFΔA (PAO1 *ΔpelFΔpslA*) + No PelF, The elution fraction, not containing PelF but containing co-eluted proteins, was added to soluble fraction of cell lysate from Pel-deficient/Psl-deficient mutant.

v) *In vitro* glycosyltransferase activity of PelF

Glycosyltransferase activity of PelF was assessed by quantifying the UDP-glucose concentrations in presence of Pel oligomers as primers and various metal cofactors. Our data showed that PelF did not show any activity when Pel oligomers were added to the reaction mixture. Similarly no activity of PelF was observed when potential metal co-factors $MnCl_2$, $MgCl_2$. and $CaCl_2$ were used in the assay (data not shown). To assess the role of undecaprenyl phosphate as an acceptor molecule, inner membrane (IM) fractions

were added to the assay reaction. UDP-glucose concentrations were not changed in the presence or absence of IM, when compared to the reaction in which PelF was not added. Interestingly, when the soluble fraction of Δ*pelF* mutant was added to the assay, PelF showed activity by reducing the UDP-glucose concentration from 53.9 ± 2.98 to 39.9 ± 2.84 nmole. When retentate fractions (>10kDa) were added to the reaction in presence of PelF, UDP glucose concentration was decreased from 57.65 ± 4.62 to 40.80 ± 2.27 nmoles. UDP-glucose concentrations did not show any significant difference in the presence or absence of PelF when filtrate (<10kDa) was added to the assay reaction (Figure 4).

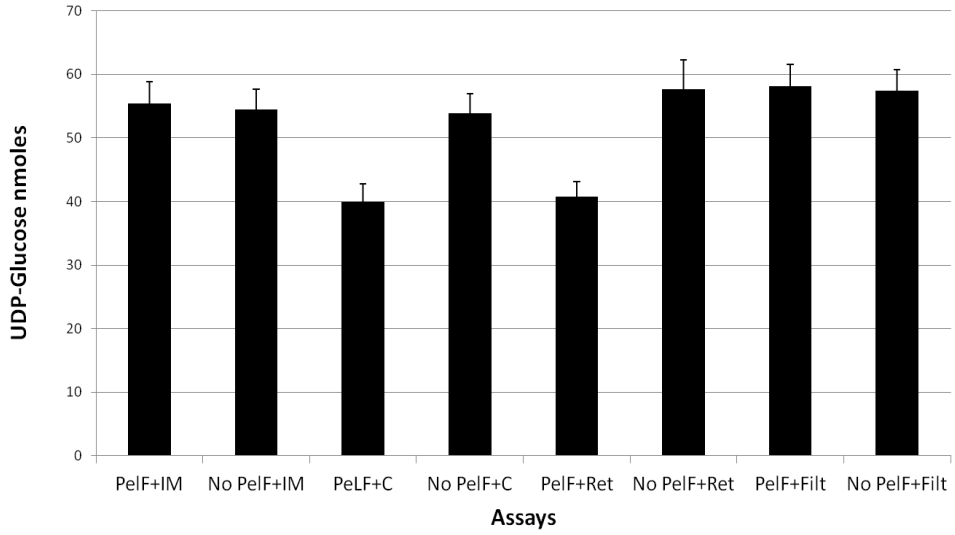


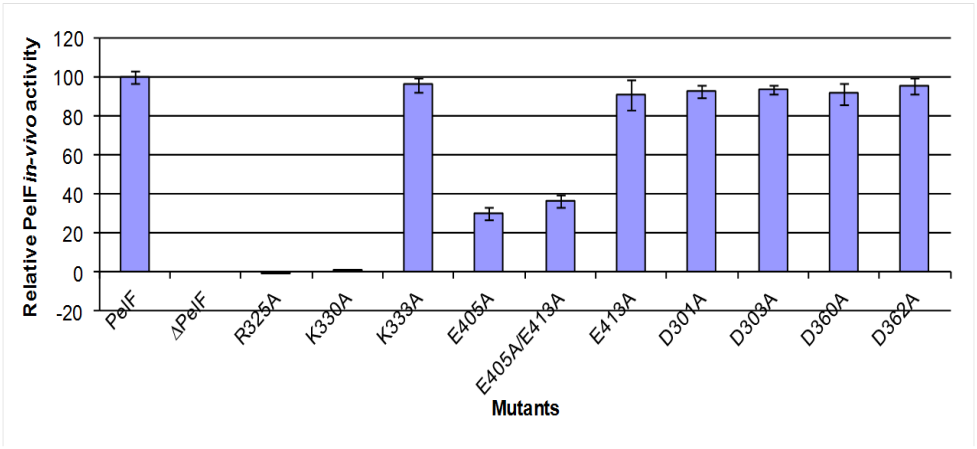
Figure 4. PelF activity as measured by UDP-glucose consumption. The residual UDP-glucose after reaction completion was determined and presented. PelF+IM, PelF and 5 µL inner membrane fraction (containing 4mg/mL total protein) was added; No PeLF+IM, partially purified suspension without PelF and 5 µL (containing 4mg/mL total protein) inner membrane fraction was added; PelF+C, PelF and 5 µL soluble fraction (containing 4mg/mL total protein) was added; No PeLF+C, partially purified suspension without PelF and 5 µL soluble fraction (containing 4mg/mL total protein) was added; PelF+Ret, PelF and 5 µL Retentate of soluble fraction filtered through filter with cut off >10kDa (containing 4mg/mL total protein) was added; No PeLF+Ret, partially purified suspension without PelF and 5 µL Retentate of soluble fraction filtered through filter with cut off >10kDa (containing 4mg/mL total protein); PelF+Filt, PelF and 5 µL filtrate of soluble fraction filtered through filter with cut off >10kDa (containing 1mg/mL total protein) was added; No PeLF+Filt, partially purified suspension without PelF and 5 µL filtrate of soluble fraction filtered through filter with cut off >10kDa (containing 1mg/mL total protein).

vi) Analysis of the catalytic mechanism of PelF by site directed mutagenesis.

To identify amino acid residues involved in the catalytic mechanism of PelF, site-specific mutagenesis of conserved residues was performed. In PelF, E405 and E413 are proposed to be the two glutamic acids of the EX_7E motif based on sequence homology to characterised GT4 glycosyltransferases. Replacement of neither E405 nor E413 abolished the activity of the enzyme. However, replacement of E405 with A, did reduce the activity of enzyme to 30.35 ± 3.15% whereas replacement of E413 with A, showed no significant effect on the activity of the enzyme as assessed by pellicle formation using the Congo red binding assay (Figure 5). The double mutant E405A/E413A showed an *in vivo* activity of 36.74 ± 3.24% of wild type. This was similar to the single mutant E405A suggesting that the second glutamic acid does not play a role in the enzymatic activity of PelF (Figure 5). The role of a conserved arginine and lysine had been shown previously in glycosyltransferases of the GT4 family. To study the role of the conserved lysine and arginine in PelF, R325, K330 and K333 were replaced by A, respectively. Interestingly, the mutant R325A or K330A was not able to form any pellicle at the air-liquid interface, comparable to PelF deficient strain. This data suggested that these amino acid residues play a critical role in PelF activity. The mutant K333A formed a pellicle similar to the wild type as the Congo red binding was 96.12 ± 3.51% of that of the wild type.

Although PelF appears to belong to GT4 family that are metal independent retaining glycosyltransferases, it does appear to have two DXD motifs, known to be involved in the catalytic activity of metal dependent glycosyltransferases. To exclude the possibility of these being involved in PelF's catalytic mechanism, all four aspartic acids were replaced by alanine, respectively (10, 11). The pellicle forming ability of the mutants D301A, D303A, D360A and D362A was 95.86 ± 3.03%, 96.39 ±2.47%, 95.21 ± 5.21% and 97.51 ± 3.86%, respectively, when compared to the wild type (Figure 5).

A



B

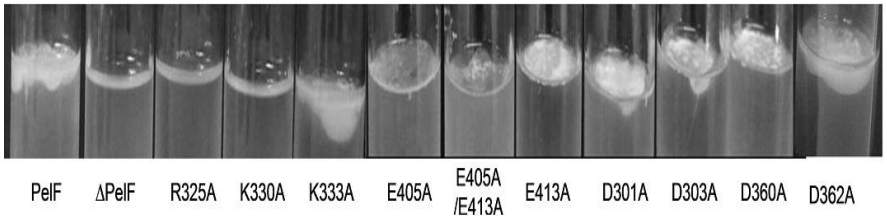


Figure 5. In vivo activity of PelF and its variants as determined by pellicle production at the air-liquid interface by various *P. aeruginosa* strains. Pellicle produced by *P.aeruginosa* strains harbouring different variants of PelF after 96-h of growth as a static cultures. A, the Congo red binding assay. Pellicle was quantified using the Congo red binding assay as described under "Materials and Methods" The percentages shown are the mean values of three independent assays. B, pellicle formation mediated by variants of PelF compared with wild type His_{10}-PelF.

DISCUSSION

Less is known about the structure and chemical nature of Pel. In this study, the production of PelF, a putative glycosyltransferase, was demonstrated and its functional role in Pel formation was assigned. Here experimental evidence for the subcellular localization of PelF and the use of UDP-glucose as substrate towards biosynthesis of the Pel polysaccharide was provided. Essential amino acid residues involved in activity of PelF were identified. PelF, tagged with deca-histadines at its N-terminus, was able to restore pellicle formation when expressed in a PelF-deficient strain suggesting that the addition of the extra amino acids at the N-terminus of PelF did not interfere with the function of the protein. Bioinformatics analysis showed that PelF is a putative glycosyltransferase with no transmembrane helices. This suggested that PelF might be soluble and located to the cytosol. Subcellular fractionation confirmed that PelF is mainly found in the soluble cytosolic fractions of cells (Figure 1).

Three proteins which were co-eluted with PelF were having apparent molecular weights similar to PelA (Molecular weight: 104.5kDa), PelE (Molecular weight: 36kDa) and PelC (Molecular weight: 18.7kDa) respectively. The absence of these proteins in the elution fraction derived from the *pelF*-deficient mutant suggested that these proteins could potentially interact with PelF. However these proteins were not found to be encoded by *pel* operon. The hypothetical protein PA4657 (GI: 15599852) in *P. aeruginosa* showed sequence similarity with FAD/NAD dependent oxidoreductases. Previously it has been shown that FAD dependent oxidoreductase are required to maintain di-sulphide bonds of bacterial proteins (26). PelF contains 6 cystein residues which may be involved in di-sulphide bond formation. It can be speculated that PA4657 could be required to maintain di-sulphide bonds of PelF.

In a previous study comparison of carbohydrate analysis of the total exopolysaccharides produced by Pel-deficient and Pel-producing strains showed that Pel might be composed of glucose (27). Hence it was assumed that UDP-glucose could be a direct precursor of Pel synthesis and was used as substrate by PelF, the only putative glycosyltransferase encoded by the pel operon. Consequently, in an isogenic *pelF* knockout strain i.e. in the absence of putatively UDP-glucose converting PelF, UDP-glucose might be present in the cytosol at elevated concentrations when compared to the wild type. For this purpose a UDP-glucose dehydrogenase based assay was developed which confirmed that the UDP-glucose levels in the *pelF* knockout mutant

were significantly elevated suggesting that UDP-glucose is substrate of PelF, a precursor of Pel and that glucose is a constituent of the Pel polysaccharide (Figure 3). UDP-glucose quantification has been done previously by capillary zone electrophoresis (CE) (28), and high-performance liquid chromatography (29). MALDI time-of-flight mass spectrometry had also been used for detection of a range of metabolites in cells (30). Here the use of UDP-glucose dehydrogenase to quantify concentration of UDP-glucose in samples of cell extracts was reported. The low K_m values of UDP-glucose dehydrogenase for UDP-glucose makes this enzyme an ideal candidate for sensitive detection of UDP-glucose (21). The UDP-glucose dehydrogenase catalyses a 2-fold oxidation of UDP-glucose (UDP-α-D-glucose) and reduces NAD(P)/NAD^+ to NADH which concentration can be easily monitored spectrophotometrically (31). Previously it has been reported that NADH does not inhibit UDP-glucose dehydrogenase by binding to its active site at higher concentrations, (32, 33). *In vitro* PelF utilized UDP-glucose as a substrate in the presence of soluble components with an apparent molecular weight >10 kDa indicating that PelF uses UDP-glucose as a donor substrate towards glycosylation of an unknown receptor molecule. In *E. coli* during O-antigen biosynthesis undecaprenyl phosphate acts as receptor (34). No PelF activity, in the presence of the inner membrane (IM) fraction suggested that PelF does not require undecaprenyl phosphate as acceptor molecule for initiation of polysaccharide biosynthesis (Figure 4). Interestingly, PelF showed activity in the presence of soluble fraction containing macromolecules with a size ≥10kDa (Figure 4). This suggested that the PelF activity requires the presence of another factor i.e. an acceptor molecule and/or an activator protein. Previously it has been shown that the activation of some glycosyltransferases depend upon their interaction with auxiliary proteins. A glycosyltransferase, DesVII, involved in the biosynthesis of macrolide antibiotics methymycin and pikromycin in *Streptomyces venezuelae* was only activated if an auxiliary protein, DesVIII was present (35-37). Similarly other glycosyltransferases have been found to require another auxiliary protein for their activity (38-40). A recent study has shown the role of two auxiliary proteins, Srm6 and Srm28, in the activation of two glycosyltransferases Srm5 and Srm29, respectively, both required for the biosynthesis of Spiramycin in *Streptomyces ambofaciens* (41). Activity of glycosyltransferase EryCIII, required for biosynthesis of the antibiotic erythromycin D, was dependent upon an auxiliary protein EryCII (42). Although the role of these

auxiliary proteins are not clear, it is proposed that these auxiliary proteins are required to induce a conformational changes of the glycosyltransferases consequently activating these enzymes (35, 41).

Based on amino acid sequence similarities, glycosyltransferases have been classified into 91 families by the CAZy database. Bioinformatics analysis suggested that PelF belongs to glycosyltransferase family 4 (CAZy) and glycosyltransferase 1 family (Pfam) respectively. Members of this family are retaining glycosyltransferases. A conserved EX_7E motif present in C-terminal domains of the retaining glycosyltransferases has been previously reported as a characteristic of these glycosyltransferases (43). An alignment of the amino acid sequence of PelF with glycosyltransferases from the GT4 family, showed the presence of conserved amino acid residues and an EX_7E motif in the C-terminal domain of the protein. Previous studies have proposed that these conserved residues are involved in stabilizing the donor substrate and in glycosidic bond formation as a nucleophile (44-49). Here it was shown that replacement of E405 in this motif with alanine caused significant reduction in PelF activity, whereas mutation of the second glutamic acid, E413, had almost no impact on PelF activity as shown by pellicle production at the air-liquid interface (Figure 5). Although according to the model proposed by Kapitonov and Robert (43) the second glutamic acid residue in the EX_7E motif is anticipated to be the catalytic residue in retaining glycosyltransferases. However it had been shown that this is not always the case. For example in AceA, a mannosyltransferase from *Acetobacter xylinum* (44), human muscle glycogen synthase (45) and Alg11, an alpha1,2-mannosyltransferase from *Saccharomyces cerevisiae* (50), replacement of first and not the second glutamic acid residue with alanine resulted into significant reduction in enzyme activity. However, in Gpi3, involved in glycosylphosphatidylinositol biosynthesis of *S. cerevisiae*, the second glutamic acid residue has been shown to be important for the enzyme activity (47). In alg11 replacement of the first glutamic acid with alanine (E405A) significantly reduced activity but complete loss of enzyme activity was not observed when compared to Δalg11 mutant. However, a double mutation (E405A/E413A) of both the first and second glutamic acid residue resulted into complete inhibition of Alg11 activity (50). Similarly in PelF, the mutation of the first glutamic acid E405A did not abolish the activity completely (Figure 5). Interestingly, a double mutation (E405A/E413A) in PelF did also not result in complete

loss of enzyme activity. This indicated that the second glutamic acid is not essential for activity and perturbation in the motif caused by replacing the second glutamic acid residue with alanine is tolerated by the enzyme, suggesting a distinct reaction mechanism which differs from other glycosyltransferases.

In some of glycosyltransferases a DXD signature is present in which the carboxyl groups coordinate a divalent cation and/or a ribose (51, 52). Two conserved DXD motifs were found in PelF. Replacing all four aspartic acid residues with alanine, respectively, showed no effect on enzyme activity, suggesting that these aspartic acid residues are not essential for the catalytic reaction mechanism as had been described for other glycosyltransferases (Figure 5).

In previous studies, a conserved arginine and/or lysine have also been shown to be required for enzyme activity of glycosyltransferases. In AceA (44) and Alg11 (50) replacement of the conserved K211 and K319 with A, respectively, significantly reduced the activity of these enzymes. Similarly, mutagenesis of conserved R604 in chitin synthase from *S. cerevisiae* has resulted in drastic decrease of the enzymatic activity (53). In PelF when the respective K330 and R325 were replaced with A, the *in vivo* enzyme activity of each mutant K330A or R325A was abolished (Figure 5). These data suggested that both K330 and R325 have an essential role in enzymatic activity of PelF. Structural analysis has shown that R196 and K202 in the mannosyltransferase PimA from mycobacteria (48) and R300 and K305 in the glycogen synthase from *E. coli* form hydrogen bonds to the oxygens of the distal phosphate group of the donor nucleotide sugar (54). Using the Protein Homology Recognition Engine PHYRE^2 (55), a model structure of PelF based on the crystal structure of WaaG from *E. coli* (PDB ID: 2IW1) was generated with 100% confidence (Figure 6). Informed by this structural model and the site-specific mutagenesis data, it is suggested that R325 and K330 in PelF are involved in forming a hydrogen bond with the phosphate oxygens of UDP-glucose. In the PelF structural model E405, R325 and K330 form a UDP-glucose binding pocket which could constitute the catalytic site of the enzyme (Figure 6).

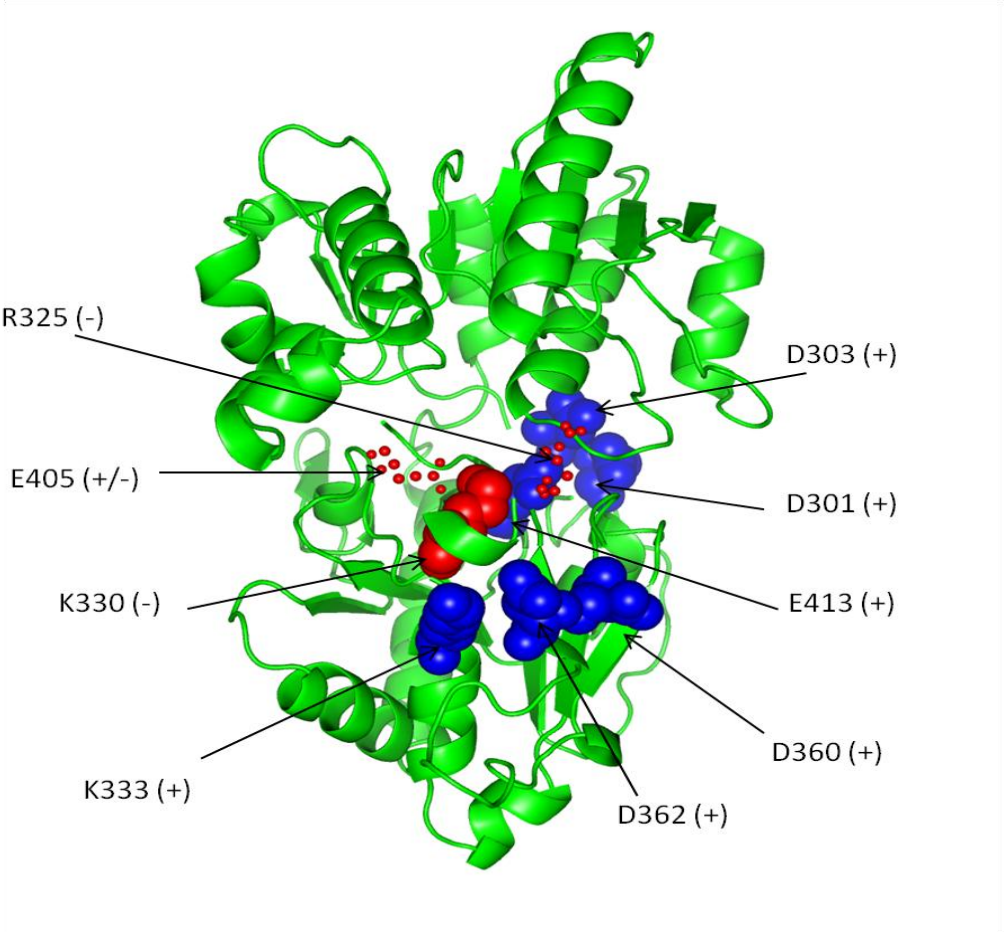


Figure 6. Structural model of PelF generated by PHYRE^2, showing localization of amino acid residues which were replaced with alanine. Selected amino acid residues were replaced with alanine using site directed mutagenesis as described in "MATERIALS AND METHODS". Residues targeted for replacement are shown as sphere. (+) indicate no impact on activity, (+/-) indicate partial loss of enzymatic activity and (-) indicate that activity of the enzyme was lost.

Overall our study suggested that PelF is a soluble glycosyltransferase that uses UDP-glucose as a donor substrate towards the biosynthesis of the Pel exopolysaccharide. Site directed mutagenesis showed that E405, the first glutamic acid of conserved EX_7E motif plays a significant but non-essential role in PelF activity and that R325 and K330 are essential for the activity of PelF.

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SUPPLEMENTARY MATERIAL

Table S1: Table of strains, plasmids and primers used in this study.

Strains, plasmids, or oligonucleotide	Description or sequence (5´-3´)	Source or reference
Strains		
E.coli JM110	*rpsL* (Strr) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F´ *traD36 proAB lacI*q*Z*Δ*M15*].	Invitrogen
E. coli TOP10	*F-mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*	Invitrogen
P. aeruginosa PAO1 Δ*pslA*Δ*pelF*	Markerless, isogenic *pslA* and *pelF* deletion double mutant derived from PAO1	(3)
P. aeruginosa PAO1 Δ*pslA*	Markerless, isogenic *pslA* deletion double mutant derived from PAO1	(3)
P. aeruginosa PAO1 Δ*pslA*Δ*alg8*	Markerless, isogenic *pslA* and *alg8* deletion double mutant derived from PAO1	(3)
Plasmids		
pBBR1MCS-5	Gm^r; broad-host-range vector; P_{lac}	(56)
pBBR1MCS-5: His_{10} - PelF	*Nde1-Xba1* fragment comprising 10-Histadine gene tagged at 5' end of the *pelF* gene inserted into vector pBBR1MCS-5	This study
Oligonucelotides		
PelF-His-Frw	AAACATATGCACCACCATCACCACCATC ACCACCATCACACCGAACACACCGCTCC GACGGCGC	
PelF Rev	AAATCTAGATCATGCAATCTCCGTGGCT TCGCGG	
PelF(His)E405-AFrwL	CAGCGCCGCGCAGCCGCTGGTGATCCTC G	
PelF(His)E405-AFrwS	AGCCGCTGGTGATCCTCG	

	GCGCGGCGCTGATCGAGGTGAGGACCA
PelF(His)E405-ARevL	TCAG
PelF(His)E405-ARevS	ATCGAGGTGAGGACCATCAG
	CCTCGCCGCCTGGGCTGCCGGCGCCCCG
PelF(His)E413-AFrwL	GTG
PelF(His)E413-AFrwS	GGGCTGCCGGCGCCCCGGTG
	AGGCGGCGAGGATCACCAGCGGCTGCG
PelF(His)E413-ARevL	CTT C
PelF(His)E413-ARevS	ATCACCAGCGGCTGCGCT TC
	CCTCGCCGCCTGGACCGGCGCCCTCGAA
PelF(His)D303-AFrwL	CGG
PelF(His)D303-AFrwS	GGACCGGCGCCCTCGAACGG
	AGGCGGCGAGGTCGATGCCGTTGGGGA
PelF(His)D303-ARevL	TCAC
PelF(His)D303-ARevS	TCGATGCCGTTGGGGATCAC
	TCCGGCCTATGCCAGCGAATGCCGCAGC
PelF(His)D362-AFrwL	CTG
PelF(His)D362-AFrwS	CCAGCGAATGCCGCAGCCTG
	CATAGGCCGGATCTTCCTCCTCCGGACC
PelF(His)D362-ARevL	GAC
PelF(His)D362-ARevS	TCTTCCTCCTCCGGACCGAC

Table S2: PelF peptides identified by MALDI-TOF-TOF

Peptide	Observed	Mr(expt)	Mr(calc)	Miss	Score	Expect	Peptide
2	1201.5825	1200.5752	1200.6251	1	59	2.3e-007	R.IGREDFLHSK.A
3	1216.5494	1215.5421	1215.5957	0	68	2.5e-008	R.YYTEALMLGR.Y
4	1227.6030	1226.5957	1226.6520	0	68	2.4e-008	R.WQAAQAVGLQR.V
7	1323.6367	1322.6294	1322.6870	0	79	1.5e-008	R.YLLSEHGIYTK.E
8	1353.5862	1352.5789	1352.6360	0	88	7.3e-011	K.ASWEAITAGYER.Y
10	1387.6714	1386.6641	1386.7255	0	105	1.6e-012	R.AANPIVALYEGNR.Q
12	1413.8033	1412.7960	1412.8615	0	79	5.8e-010	R.RPPGIPPVVGLVGR.V
14	1484.7013	1483.6940	1483.7630	0	94	2e-011	R.AGEVVAIADPQATSR.A
15	1521.6864	1520.6791	1520.7479	0	117	2e-013	R.SMQAPVFMLAEAAR.R
16	1537.6743	1536.6670	1536.7428	0	(52)	3.3e-007	R.SMQAPVFMLAEAAR.R + Oxidation (M)
17	1553.6725	1552.6652	1552.7377	0	(29)	5.7e-005	R.SMQAPVFMLAEAAR.R + 2 Oxidation (M)
18	1600.7485	1599.7412	1599.8078	1	29	0.00021	R.VERYYTEALMLGR.Y
19	1614.7512	1613.7439	1613.8008	1	21	0.00039	R.ELIEGADAEDRALGR.A
20	1839.8754	1838.8681	1838.9526	0	142	3.1e-016	R.VIPNGIDLDAWTGALER.R
23	1968.8033	1967.7960	1967.8876	0	106	1.2e-012	R.YCTDPSFVNYFWTLR.S +Carbamidomethyl(C)
25	2090.0012	2088.9939	2089.0812	0	106	1.3e-012	R.MLHSISTGYAGLLGCILQR.R+Carbami (C)
28	2733.1775	2732.1702	2732.2969	0	176	1.8e-018	R.FFHYPETPDVEEGDALLDLLAEGR.I
33	3231.4546	3230.4473	3230.5731	0	127	9.8e-015	K.IDLAQANWIAENPDEQLSTGLDAEVSYIR.R
34	3327.4819	3326.4746	3326.6108	0	127	9e-015	R.HYPIPDNVLHIEEHFLETAWSSPNPQTR.Q
35	3359.5237	3358.5164	3358.6680	1	159	6.5e-018	R.KIDLAQANWIAENPDEQLSTGLDAEVSYIR.R
36	3515.6355	3514.6282	3514.7691	2	61	1.5e-007	R.KIDLAQANWIAENPDEQLSTGLDAEVSYIRR.L

Table S3: Sequences of synthetic DNA fragments used in this study.

D301A

*CCCGGG*TGATCCCCAACGGCATCGCCTCGATGCCTGGACCGGCGCCCTCG
AACGGCGGCCGCCGGGGATTCCGCCGGTGGTCGGGCTGGTCGGCCGGGTAG
TGCCGATCAAGGACGTGAAGACCTTCATCCGCGCCATGCGCGGGGTGGTCA
GCGCGATGCCGGAGGCGGAGGGCTGGATCGTCGGTCCGGAGGAGGAAGAT
CCGGACTATGCCAGCGAATGCCGCAGCCTGGTGGCCAGCCTCGGC*CTGCAG*

D360A

*CCCGGG*TGATCCCCAACGGCATCGACCTCGATGCCTGGACCGGCGCCCTCG
AACGGCGGCCGCCGGGGATTCCGCCGGTGGTCGGGCTGGTCGGCCGGGTAG
TGCCGATCAAGGACGTGAAGACCTTCATCCGCGCCATGCGCGGGGTGGTCA
GCGCGATGCCGGAGGCGGAGGGCTGGATCGTCGGTCCGGAGGAGGAAGCT
CCGGACTATGCCAGCGAATGCCGCAGCCTGGTGGCCAGCCTCGGC*CTGCAG*

R325A

acagat*CCCGGG*TGATCCCCAACGGC**ATCGAT**CTCGACGCCTGGACCGGCGCC
CTCGAACGGCGGCCGCCGGGGATTCCGCCGGTGGTCGGGCTGGTCGGCGCC
GTAGTGCCGATCAAGGA***T***GTGAAGACCTTCATCCGCGCCATGCGCGG***T***GTG
GTCAGCGCGATGCCGGAGGCGGA***A***GGCTGGATCGTCGGTCCGGAGGAGGA
AGACCCGGACTATGCCAGCGAATGCCGCAGCCTGGTGGCCAGCCTCGGC*CT*
*GCAG*gacaagg

K330A

acagat*CCCGGG*TGATCCCCAACGGC**ATCGAT**CTCGACGCCTGGACCGGCGCC
CTCGAACGGCGGCCGCCGGGGATTCCGCCGGTGGTCGGGCTGGTCGGCCGG
GTAGTGCCGATCGCCGA***T***GTGAAGACCTTCATCCGCGCCATGCGCGG***T***GTG
GTCAGCGCGATGCCGGAGGCGGA***A***GGCTGGATCGTCGGTCCGGAGGAGGA
AGACCCGGACTATGCCAGCGAATGCCGCAGCCTGGTGGCCAGCCTCGGC*CT*
*GCAG*gacaagg

K333A

acagat*CCCGGG*TGATCCCCAACGGC**ATCGAT**CTCGACGCCTGGACCGGCGCC
CTCGAACGGCGGCCGCCGGGGATTCCGCCGGTGGTCGGGCTGGTCGGCCGG
GTAGTGCCGATCAAGGA***T***GTGGCCACCTTCATCCGCGCCATGCGCGG***T***GTG
GTCAGCGCGATGCCGGAGGCGGA***A***GGCTGGATCGTCGGTCCGGAGGAGGA
AGACCCGGACTATGCCAGCGAATGCCGCAGCCTGGTGGCCAGCCTCGGC*CT
GCAG*gacaagg

Underlined and italicized: *Pst*I and *Sma*I restriction sides respectively

Underlined: Nucleotide changes to introduce site-directed mutations

Italicized and Bold Silent mutations introduced to adjust the CG content which was problematic during DNA synthesis.

Bold: Silent mutations introduced to add a new restriction site i. e *Cla*I.

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CHAPTER VI

General Discussion

GENERAL DISCUSSION

The microbial biofilm matrix is composed of extracellular polysaccharides, proteins (1, 2), and extracellular DNA (3). A few studies have also proposed a role for dead cells in the architecture of the biofilm matrix (4). The *Pseudomonas aeruginosa* biofilm is not an exception and its matrix is comprised of the aforementioned components (5). Three important exopolysaccharides, (Pel, Psl, and alginate), produced by *P. aeruginosa*, have shown their importance in biofilm formation (5, 6) .

Psl plays a crucial role in biofilm formation and architecture. Results in the study undertaken showed that all Psl-deficient but Pel-producing and/or alginate-producing mutants had impairment in initial attachment with the surface (Chapter III) suggesting that alginate and Pel have little role in initial attachment. These results are consistent with previous studies which showed the significance of Psl in initial attachment of the cells to an abiotic surface in biofilm formation (7-9). Additionally, it has been shown that an enhanced Psl production leads to enhanced cell-surface attachment (8). *P. aeruginosa* PAO1 cells start producing Psl during planktonic growth (7). This newly synthesized Psl is arranged on the cells in helical-like patterns which assist in cell-cell and cell-surface attachment (10). The mechanism of Psl-surface interaction is not known. Previous studies have suggested that bacterial cells are initially attached reversibly to surfaces by hydrophobic cell-surface interactions (11, 12). In contrast, the production of exopolysaccharides and other adhesins help bacteria attach irreversibly to hydrophilic surfaces (11-13). In this study, lack of attachment in the absence of Psl indicates that it may act as an adhesin in *P. aeruginosa* and is required for irreversible attachment. Additionally, it has been recently demonstrated that Psl stimulates the production of c-di-GMP Psl acts as a signal to stimulate two DGCs, SiaD and SadC, consequently c-di-GMP intracellular level is elevated. This leads to the enhanced production of Psl and other components of the biofilm (14). C-di-GMP is a secondary messenger required for irreversible attachment and biofilm initiation [reviewed in. (15)(15)]. Therefore, inability to produce Psl may result in impaired c-di-GMP biosynthesis, negatively affecting biofilm formation. Psl also plays a post-attachment role in development of the biofilm architecture (10). The results of the study undertaken showed that Psl deficient mutants were not able to form typical mushroom-like structures, instead a flat multilayer biofilm was formed (Chapter III). Recently, it has been shown that Psl is distributed in the biofilm periphery which maintains the

mushroom-like biofilm architecture (10). Psl has also been recognised as a cell-cell interconnecting polymer (8, 16). In this study, experimental data showed that the expression of the *psl* operon was high in the periphery of PAO1 biofilm (Chapter IV). In the light of all the above, it can be speculated that bacterial cells on the surface of biofilm produce more Psl. These cells, connected through Psl, make an outer boundary 'wall' of the biofilm and contribute to the mushroom-like architecture of the *P. aeruginosa* PAO1 biofilm. In our study, Psl deficient mutants formed thick, flat, and multilayer biofilms after 96 h of growth (Chapter III) whereas a previous study by Ma *et al*. showed that Psl deficient *P. aeruginosa* mutants were not able to form multilayer biofilms (8). This discrepancy in results is most probably due to Ma *et al.* observing the biofilm after 24 h of growth, whereas observations in this study were recorded after 96 h of growth. The Psl-deficient mutants may use eDNA as cell-cell interconnecting polymer. Previously eDNA has been shown to act as a cell-cell and cell-surface interconnecting polymer at the early stages of biofilm development (3). A few cells which might be attached irreversibly to the surface with the help of eDNA may start producing Pel, resulting in delayed biofilm formation (Chapter III). However, these biofilms are flat and do not show typical mushroom-like structures (Chapter III). The role of eDNA is further strengthened by the results showing the presence of increased amount of dead cells and extracellular DNA in such biofilms (Chapter III).

The exopolysaccharide Pel is important for attachment and biofilm development in various strains of *P. aeruginosa* (17, 18). However, its role has been mostly studied in *P. aeruginosa* PA14 which is a Psl-deficient strain and mainly depends on Pel for biofilm formation (19-22). Previous study revealed that Pel is important for formation of a pellicle at air-liquid interfaces (19) and also for attachment (20) in PA14. This study, has explored the role of Pel in PAO1 biofilms (Chapter III). Solid surface attachment assays showed that Pel has no role in the initial attachment but plays a crucial role at later stages of biofilm development especially in the absence of Psl (Chapter III). A previous study has demonstrated that Pel-mediated antibiotic resistance was only seen in *P. aeruginosa* biofilms and not in planktonic cells and showed Pel was only expressed in biofilm-forming cells (20). Experiments conducted in present study to observe temporal regulation of the Pel biosynthesis operon in the biofilm showed that the *pel* expression in biofilms was initiated late and increased with the age of the biofilm (Chapter IV). This could be due to the transcription factor, FleQ, which

represses the *pel* promoter. FleQ is inactivated only when adequate amounts of c-di-GMP are produced in biofilm-forming cells and c-di-GMP binds to FleQ derepressing the *pel* promoter (23, 24). Pel-deficient Psl-producing mutants of PAO1 formed mushroom-like biofilms but with lesser compactness when compared to the wild type (Chapter III). This suggested that Pel is dispensable for PAO1 biofilm formation especially in the presence of Psl. Consistent with these results, a recent study has shown that Pel has a redundant role in PAO1 biofilm formation (25). In contrast to PAO1, an alginate overproducing mutant PDO300 was not able to maintain a biofilm in the absence of Pel, suggesting a different role of Pel in mucoid strain biofilms (Chapter III). What makes Pel vital for maintaining PDO300 biofilm? Interestingly, other studies showed that the alginate overproducing mutants FRD1 and PDO300 produced less Psl as compared to the non-mucoid strain PAO1 (26, 27). This suggested that in PDO300 Pel was essential for forming and maintaining the biofilm. Because the mucoid strains of *P. aeruginosa* have been found responsible for resistant infections in patients with lung infections (28), therefore, it is imperative that all attempts to control *P. aeruginosa* biofilms must take Pel in account.

In the past, it has been suggested that reversibly-attached cells were attached to surfaces through one pole (29). In this study, Psl/Pel deficient (alginate producing) cells were attached to the surface through one pole (Chapter III) indicating that alginate alone in the absence of Psl and Pel is not capable of attaching cells irreversibly to surfaces. These results are consistent with previous studies in which it has been shown that alginate expression is not required for the initial steps of biofilm development in PAO1 (30). Furthermore, alginate-deficient PAO1 showed no difference in terms of biofilm development after 8 h of incubation when compared to wild type PAO1 (30). Moreover, alginate plays a negative role in bacterial attachment and overproduction of alginate further decreases the ability of cells to attach to the surface (31, 32). Involvement of alginate in the architecture of non-mucoid strain biofilms is controversial. Previously, in non-mucoid strain-based biofilms, alginate was detected using alginate-specific antibodies (33, 34). In contrast, the data from a number of microarray experiments showed alginate biosynthesis genes were not expressed under similar conditions (35-37). In addition, Wozniak *et al*. also observed that the presence or the absence of alginate did not affect the architecture of non-mucoid biofilms (30). Interestingly, this study has demonstrated the role of alginate in the non-mucoid, wild type PAO1

biofilms. The result showed that wild type formed structured biofilms whereas the mutant PAO1Δ*alg8* formed flat biofilms (Chapter III). This discrepancy of results could be due to the use of different growth medium. Furthermore, the significance of alginate in *P. aeruginosa* biofilms architecture are demonstrated by previous study which has shown that an increase in alginate plays a pivotal role in shaping the structure of biofilm in alginate overproducing mutants (31).

Significance of eDNA has been previously demonstrated in biofilms formed by Gram-positive as well as Gram-negative bacteria (38, 39). It was established that eDNA was required for maintaining the 3D biofilm architecture and cell-cell connections (3). eDNA has been shown essential for initial attachment of the cells to form biofilm and no mature biofilm could be formed when developing biofilms were treated with DNase at early stage, However, DNase traetment at later stage was not essentially required to maintain the 3D architecture of the biofilm (3). In addidtion to the role of eDNA in architecture of the biofilm, a recent study has suggested that eDNA plays a vital role in bacterial motility. eDNA produced by dead cells is used by bacterial cells as trail for their movement and this help them to self organise their architecture (40). eDNA is such an important component of the biofilm that bacterial species have evolved differnt procedure to release DNA. Prophage-mediated cell death has been reported as a source of eDNA in biofilms (4, 41). Other sources of eDNA include quorum sensing regulated release of DNA (38) and outer membrane vesicles (OMV) released from living *P. aeruginosa* cells (42, 43). This study has demonstrated that eDNA release increases in the absence of alginate. Results showed that mutants deficient in alginate produced relatively more dead cells and eDNA when compaed to wild type (Chapter III). Alginate, an anionic polysaccharide, is an important matrix component which protects bacterial cells in biofilms from cationic antimicrobial peptides (CAPs) produced by the host's cells (44). Recently, a similar role has been reported for eDNA (45). This study suggests that bacterial biofilms increases the production/release of eDNA in the absence of alginate to compensate for the defensive role of alginate.

Interestingly, it was found that overproduction of exopolysaccharides in *P. aeruginosa* decreases the production of other exopolysaccharides. In the absence of Psl, *P. aeruginosa* cells produced more Pel, and, in the absence of Pel, more alginate was produced (Chapter III). This exopolysaccharide biosynthesis could have been regulated at the transcriptional and/or biosynthesis level. Recently it has been observed that the

control of exopolysaccharide production occurs at the transcriptional level (26). That study found that the global regulator, AlgU, which regulates the *algD* promoter and upregulates the alginate biosynthesis (46), also increased the biosynthesis of another transcription regulator, AmrZ. Consequently, AmrZ down regulated the transcription of the *psl* operon and hence decreased Psl production (26). Our study also showed that, in PDO300, in which AlgU is unleashed due to a mutational inactivation of MucA, *psl* transcription was 30% less than PAO1 in which AlgU is post-translationally inactivated due to functional MucA (Chapter III). In addition, the *psl* operon expression was found very much less in PDO300 when compared to PAO1 (Chapter IV). Exopolysaccharide production may also be regulated at the post-transcriptional level. A recent study has shown that synthesis of different exopolysaccharides produced by *P. aeruginosa* is also post-transcriptionally regulated (27). The study showed that excessive production of Psl can decrease the production of alginate, and that Pel overproduction might lead to decreased Psl production due to the limited availability of common precursors (27).

The importance of exopolysaccharides in the biofilm formation and architecture has been well studied using *in vitro* biofilm studies. In a recent study it was shown that these exopolysaccharides are also important for biofilm development *in vivo*. Pel deficient mutant, *pelB::lux* was not able to form biofilm in *Drosophila melangaster*. However, the RsmA deficient mutant (which is a high Pel and Psl producer as compared to wild type PAO1) formed thick and dense biofilms (47).

This study also highlighted the important role of Pel in PAO1 biofilm. It was observed that in the absence of Psl and alginate, Pel took the responsibility for biofilm development in *P. aeruginosa* grown under laboratory conditions. Therefore, it is important to understand the biosynthesis of Pel. In this study the role of PelF, the only glycosyltransferase encoded by the *pel* operon, in the biosynthesis of Pel has been studied. Results from this study showed that PelF is essential for the biosynthesis of Pel and no other enzyme can compensate for the role of PelF.

Unlike the *psl* operon, which encodes for four glycosyltransferases (6), there is only one putative glycosyltransferase encoded by the *pel* operon which suggested that Pel is a homopolymer. Recently, on the basis of bioinformatics analysis, Franklin *et al*. have also suggested that Pel is a homopolymer which is synthesized in Wzy-independent manner (6). However, the chemical structure of Pel has not yet been resolved. This study shows for the first time that PelF utilizes UDP-glucose as substrate

suggesting that Pel might be composed of glucose (Chapter V). PelF was only active in the presence of the soluble fraction of cell lysate which indicated that some other component from the soluble fraction is required for activity (Chapter V). It has also been proposed that Pel is a cellulose-like polymer as pellicles formed by *P. aeruginosa* PA14 could be digested by cellulase (19). The glycosyltransferase BcsA1, involved in cellulose biosynthesis in *Acetobacter xylinum,* (48), requires c-di-GMP binding for its activation (49, 50). It was thought that PelF activity could be regulated by c-di-GMP but results demonstrated that PelF did not show activity in the presence of the soluble fraction free of >10kDa macromolecules (Chapter V). This suggested that PelF activity is not dependent upon c-di-GMP. A previous study has also shown that PelF does not have a PilZ domain and does not bind c-di-GMP (51). Some glycosyltransferases require divalent cations for their activity [reviewed in. (52)(50)]. However, PelF belongs to the GT4 family (the CAZy database) and members of this family do not depend upon divalent cations for their activity. Results also showed that PelF activity was not dependent on the divalent cations tested in this study (Chapter V). In addition, DXD motifs present in glycosyltransferases interact with divalent cations for their activity. In this study perturbation of DXD motifs had no impact on enzyme activity. Previous studies have shown the importance of EX_7E motif in retaining glycosyltransferases (53). In most glycosyltransferase protein sequences, both of the glutamic acids residues were conserved and were suggested to be the catalytic residues (53-55). Results showed that the first glutamic acid played a role in PelF activity but the activity was not completely dependent upon this glutamic acid residue (Chapter V). A role for conserved arginine and lysine residues has been shown previously in some enzymes of the GT4 family (55-57). Interestingly, this study found that one conserved lysine (K 330) and one arginine (R325) were essentially required for the activity of PelF (Chapter V). The R-groups of conserved arginine and lysine residues have been shown to interact with the distal phosphate of nucleotide sugars and are thought to be required to stabilize the substrate (58, 59)

Overall, this study has explored the role of three important exopolysaccharides and eDNA in the architecture of *P. aeruginosa* biofilms. With the help of mutants able to synthesize single, double, or all three polysaccharides, this study has shown the impact of one exopolysaccharide production on the biosynthesis of other polysaccharides. In addition, experiments conducted to observe the temporal-spatial

regulation of exopolysaccharides in biofilms gave an insight into the post-attachment steps of biofilm development. This study showed Pel's role in PAO1 and PDO300 biofilms which had been previously overlooked. Keeping in mind the importance of Pel, the study assigned the role of PelF as a glycosyltransferase in the biosynthesis of Pel, and initiated the process of understanding the mechanism of Pel biosynthesis in *P. aeruginosa* PAO1.

FUTURE OUTLOOK

In this study the role of exopolysaccharides in the architecture of biofilm has been studied. A battery of mutants, developed in this study, has been used to explore the role of exopolysaccharides in biofilm *in vitro*. In the future these mutants could be used to investigate the role of these exopolysaccharides in pathogenesis of disease *in vivo,* using a mouse model. Biofilms are resistant to antibiotics and that how these exopolysaccharide contribute towards this resistance can also be further investigated. These mutants can also be used to study the effects of exopolysaccharides on the immune responses of the host.

Temporal and spatial regulation of the exopolysaccharide biosynthesis operons has been investigated in wild type PAO1 and engineered PDO300 strains. The mutants and plasmids harbouring promoter-reporter fusions generated in this study can be employed to probe the effects of one exopolysaccharide on the expression of other exopolysaccharides. Further investigation is required to discover the PelF acceptor molecule. Attempt could be made to crystallize PelF, enabling its structure to be resolved. The chemical structure of the exopolysaccharide Pel is yet to be resolved. Pel-only producing mutants (Psl-deficient, alginate-deficient) may be used to purify Pel and subjected to analytical study. Four out of seven proteins encoded by the *pel* operon have not been experimentally studied and their role in Pel biosynthesis needs to be investigated.

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