The Biofilm Blueprint: Exopolysaccharide Form and Function in Bacterial Biofilms

Shelby L. Cole & Laura K. Jennings

Chapter 1
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Abstract This chapter examines the composition of exopolysaccharides within the bacterial biofilm matrix, unraveling their intricate roles in biofilm formation and infection pathogenesis. Serving as key structural components of the biofilm matrix, exopolysaccharides form part of the complex network of polymers secreted by biofilm bacteria. Here, we explore biosynthesis pathways, regulatory mechanisms, and multifaceted functions of exopolysaccharides—drawing insights from notable exopolysaccharides like alginate, Pel, Psl, cellulose, and poly-*N*-acetylglucosamine (PNAG). Specifically, we focus on exopolysaccharide-mediated mechanisms of adhesion, biofilm mechanics, immune evasion, and resistance to antimicrobials. Furthermore, we discuss the translational potential of antibacterial therapeutics that target exopolysaccharides. Through an exploration of the structure and functions of exopolysaccharides, this chapter contributes to a more comprehensive understanding of biofilm biology, highlighting unresolved questions and potential avenues for future research in this critical area.

Keywords Exopolysaccharides · Biofilm matrix · *P. aeruginosa* · Pel · Psl · Alginate · PNAG · Cellulose · Antibiotic tolerance

1.1 Introduction to the Biofilm Matrix

Bacterial cells rarely grow alone. In the environment and at sites of infection, bacteria often grow as biofilms or aggregates of bacteria embedded in a self-produced polymer-rich matrix. Biofilms are frequently associated with acute and chronic infections including otitis media, urinary tract infections, infectious kidney stones,

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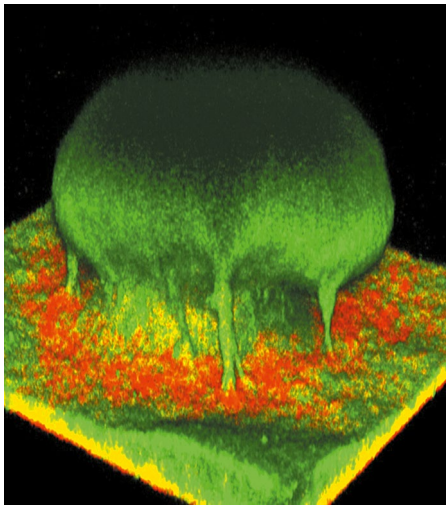

Fig. 1.1 Exopolysaccharides contribute to the form and function of the biofilm matrix. Distinct three-dimensional architecture of *P. aeruginosa* PAO1 Δ*wspF* Δ*psl* P_{BAD} *pel* biofilm overproducing the Pel exopolysaccharide

bacterial endocarditis, indwelling medical devices, chronic wounds and cystic fibrosis (CF) lung infections (Parsek and Singh 2003; Lebeaux et al. 2014). Once a biofilm infection is established, it can be incredibly difficult to eradicate. Biofilm infections tend to persist despite a robust immune response and repeated antibiotic treatment (Costerton et al. 1999; Stewart 2002). The recalcitrance of biofilm infections poses a specific threat to human health. Many bacteria exhibit multiple mechanisms of biofilm formation. This redundancy in aggregation mechanisms suggests that biofilm formation may be important to bacterial survival and pathogenicity in hostile environments.

One of the hallmarks of bacterial biofilms is the self-produced extracellular matrix which provides three-dimensional structure and protection for resident bacterial cells. The biofilm matrix is composed of extracellular polysaccharides (exopolysaccharides), proteins, nucleic acids, and other biopolymers. Exopolysaccharides are among the most abundant components of the biofilm matrix and are intricately linked to biofilm form and function (Fig. 1.1) (Flemming et al. 2007; Flemming and Wingender 2010; Sutherland 2001). Understanding the structure and function of exopolysaccharides within the biofilm matrix is a major area of research interest and may facilitate the design of effective therapeutics to disrupt biofilms.

1.2 Building Blocks of Biofilms: Diversity and Common Themes in Exopolysaccharide Structure Among Bacteria

The structure of an exopolysaccharide dictates the chemical and physical properties of the polymer, and ultimately its function in biofilms and infection pathogenesis. In the simplest sense, polysaccharides are sugar monomers linked to form a polymer.

However, exopolysaccharide structure can vary in monomer composition, glycosidic linkage, branching, sugar modifications, molecular weight, and charge (Limoli et al. 2015). The potentially unlimited combinations of monomers and linkages has the possibility to generate enormous diversity in exopolysaccharide structure; yet, only a small subset of these exopolysaccharides are utilized by bacteria for biofilm formation (Flemming et al. 2023). Some common polysaccharides utilized by diverse bacteria include alginate, Psl, Pel, cellulose, and poly-*N*-acetylglucosamine (PNAG) (Table 1.1).

Alginate is a negatively charged polysaccharide composed of mannuronic acid (ManA) and guluronic acid (GulA) (Evans and Linker 1973). Psl is a neutral polysaccharide composed of a mannose (Man), glucose (Glc), and rhamnose (Rha) in a pentasaccharide repeat (Byrd et al. 2009). Cellulose is a homopolymer composed of glucose that may include phosphoethanolamine modifications (Wang et al. 2019; Thongsomboon et al. 2018). Pel and PNAG are positively charged polysaccharides composed of partially acetylated galactosamine (GalNAc) and glucosamine (GlcNAc), respectively (Jennings et al. 2015; Mack et al. 1996).

In addition to variations in chemical structure, exopolysaccharides of the biofilm matrix can vary depending on the bacterial species, strain, and growth conditions. Many biofilm-forming bacteria produce multiple exopolysaccharides with different properties as in *Escherichia coli* and *Pseudomonas aeruginosa* which both produce multiple exopolysaccharides that vary by charge (Jennings et al. 2015; Whitfield et al. 2015). Colvin et al. found significant strain-to-strain variability in Pel and Psl polysaccharide production in clinical and environmental isolates of *P. aeruginosa* with some isolates producing both polysaccharides, while others producing only one (Colvin et al. 2012). In PA14, which harbors a mutation in Psl, Pel is crucial for biofilm formation and acts as the primary structural scaffold initiating and maintaining cell-to-cell interactions in the biofilm (Colvin et al. 2011). In contrast, Psl is the primary matrix polysaccharide in PAO1, which produces both Pel and Psl, but deletion of Pel from this strain does not drastically affect biofilm formation. Greater Pel production occurs in the absence of Psl, suggesting that Pel and Psl regulation may be linked (Ghafoor et al. 2011; Jennings et al. 2015). Furthermore, commensal and pathogenic strains of *E. coli* vary in their ability to produce cellulose and other extracellular matrix components (Bokranz et al. 2005; Da Re and Ghigo 2006). Redundancy in exopolysaccharide production may provide cells an adaptive advantage, enabling biofilm formation under changing conditions.

Indeed, cells can adapt to changing culture conditions by modifying their exopolysaccharides (Toyofuku et al. 2016). Whitfield et al. demonstrated that exopolysaccharide modifications including alginate acetylation and epimerization varied depending on growth conditions (Whitfield et al. 2015). In *E. coli*, the growth media affects cellulose production and rugose colony morphology (Serra et al. 2013a). Thus, culture conditions influence biofilm formation and exopolysaccharide production. It is thought that the flexibility to produce different exopolysaccharides between and among bacterial strains may facilitate biofilm formation and survival in a variety of environments (Ma et al. 2022).

Table 1.1 Common exopolysaccharides produced by biofilm-forming bacteria

Polysaccharide	Organism(s)	Linkage	Sugar composition	Modifications	Charge	Molecular weight (kDa)	References
Alginate	*P. aeruginosa, P. fluorescens*	β-1,4	ManA, GulA	*O*-acetylated	Anionic	300–1300	Evans and Linker (1973), Das (2022), Hay et al. (2010)
Cellulose	*E. coli, Salmonella*	β-1,4	Glc	Phosphoethanolamine	Neutral	~1000	Wang et al. (2019), Thongsomboon et al. (2018), Das (2022)
Pel	*P. aeruginosa*	α-1,4	GalNAc	50% *N*-deacetylated	Cationic	Cell assoc: >80, non-cell assoc: 0.5	Jennings et al. (2015), Le Mauff et al. (2022), Jennings et al. (2021)
PNAG	*S. aureus, S. epidermidis, E. coli, Y. pestis, A. baumannii*	β-1,6	GlcNAc	10–20% *N*-deacetylated	Cationic	Cell assoc: >460, non-cell assoc: 21	Mack et al. (1996), Maira-Litrán et al. (2002)
Psl	*P. aeruginosa, P. fluorescens*	β-1,3 α-1,2 α-1,3	Man, Glc, Rha	N/A	Neutral	Cell assoc: >10, non-cell assoc: 3-6	Byrd et al. (2009)

Abbreviations: ManA, D-mannuronic acid; GulA, L-guluronic acid; Glc, D-glucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Man, D-mannose; Rha, L-rhamnose; assoc., associated

1.3 Biosynthesis and Modification of Bacterial Exopolysaccharides

Chemically distinct exopolysaccharides share similarities in biosynthesis pathways (Das 2022). For many exopolysaccharides, biosynthesis is a multistep process consisting of (1) synthesis of activated sugar precursors, (2) polymerization, (3) polymer modification, and (4) export to the extracellular space (Gheorghita et al. 2023). Exopolysaccharides are synthesized from activated sugar nucleotide precursors using glycosyltransferases for polymerization (Low and Howell 2018; Franklin et al. 2011). Glycosyltransferases transfer the sugar moiety from the activated nucleotide sugar to an acceptor. Glycosyltransferase activity is often regulated through binding to the common signaling molecule and secondary messenger cyclic diguanosine monophosphate (c-di-GMP) (Poulin and Kuperman 2021). Typically, glycosyltransferases exhibit specificity to a single sugar precursor. Interestingly, an exception to this rule comes from *Streptococcus thermophilus* where a promiscuous glycosyltransferase can utilize galactose or *N*-acetylgalactosamine (GalNAc) for polymer synthesis (Stingele et al. 1999). In Gram-negative bacteria, the polymer is transported across the inner membrane to the periplasm during synthesis, while in Gram-positive bacteria, the polymer is transported directly to the extracellular space (Das 2022; Whitfield et al. 2015). Following polymerization, polysaccharides can be modified by acetylation, deacetylation, or epimerization (Flemming et al. 2023). Polysaccharides can remain associated with the cell through binding the cell membrane or may be non-cell associated (Ostapska et al. 2018). Some exopolysaccharides including PNAG, Pel, and Psl contain two forms: a high-molecular weight, cell-associated polymer and a low-molecular-weight, non-cell-associated form (Jennings et al. 2015; Byrd et al. 2009; Rohde et al. 2010; Maira-Litrán et al. 2002). Although the biological significance of having two forms of the polysaccharides is not clear, the extracellular form may aid in immune evasion by binding antibodies and preventing antibody-mediated killing at the bacterial cell surface (Cerca et al. 2007).

1.3.1 Cationic Exopolysaccharides from the Deacetylation of Amino Sugars: A Common Thread in Diverse Bacterial Species

One common post-polymerization modification of exopolysaccharides is *N*-deacetylation of hexosamine sugars. Deacetylation of amino sugars by deacetylase enzymes renders them positively charged (Ostapska et al. 2018). The cationic charge is important for the polysaccharide's function in biofilms and host–pathogen interactions. For instance, the cationic charge controls the adhesive properties of the polymer including biofilm formation and adhesion to host cells/polymers and abiotic surfaces (Vuong et al. 2004a; Colvin et al. 2013; Jennings et al. 2015), immune

evasion (Vuong et al. 2004a) and virulence (Vuong et al. 2004a; Subashchandrabose et al. 2013). Moreover, cationic polysaccharides can also protect bacteria from antimicrobials through repulsion and sequestration (Jennings et al. 2021; Farber et al. 1990).

Many biofilm-forming bacteria produce an extracellular matrix containing positively charged exopolysaccharides (Ostapska et al. 2018). One of the most prevalent is poly-β-1,6-*N*-acetylglucosamine (PNAG, formerly polysaccharide intercellular adhesin, PIA), produced by a broad range of Gram-negative and Gram-positive bacteria including *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Helicobacter pylori* (Cywes-Bentley et al. 2013). Notably, one of the few pathogenic bacteria that does not appear to produce PNAG is *P. aeruginosa*. Instead, *P. aeruginosa* produces a similarly positively charged exopolysaccharide called Pel composed of partially acetylated α-1,4-*N*-acetylgalactosamine sugars (Le Mauff et al. 2022; Jennings et al. 2015). Many Gram-negative pseudomonadota (formerly proteobacteria) and Gram-positive bacilli, clostridia, streptococci, and actinobacteria encode operons that share sequence similarity to Pel suggesting that the exopolysaccharide may also be widespread across phylogenetically diverse species (Whitfield et al. 2020b; Whitfield and Howell 2021). The ability to produce a positively charged amino sugar exopolysaccharide appears to be broadly conserved among biofilm-forming bacteria.

1.3.2 Role of Alginate Modifications in Biofilm Resiliency

Alginate is synthesized as a non-acetylated polymer and subsequently *O*-acetylated in the periplasm. The degree of *O*-acetylation of the mannuronic acid residues is a physical/chemical property of alginate that strongly affects its function in biofilms. Removal of *O*-acetyl groups results in increased binding of divalent cations such as calcium and reduced polymer solubility (Skjåk-Bræk et al. 1989). *O*-acetylation of alginate increases tolerance to antibiotics and enhances resistance to the innate immune system. The water binding capacity of the polymer increases with *O*-acetylation, which in turn influences its solubility, viscosity, and gelling properties. One study found that alginate protects *P. aeruginosa* biofilms from IFN-γ-mediated macrophage killing (Leid et al. 2005). Another study showed that *O*-acetylation of alginate helps *P. aeruginosa* evade the host immune system by preventing opsonization (Pier et al. 2001). The degree of *O*-acetylation can vary widely among *P. aeruginosa* isolates (Pugashetti et al. 1982). Overall, the collective work emphasizes the importance of *O*-acetylation of alginate in resistance to antimicrobials and protection from the host immune system.

1.4 Overview of Exopolysaccharide-Mediated Functions in the Biofilm Matrix

1.4.1 Sticky Business: The Adhesive Role of Exopolysaccharides

The fate of cells within biofilms—that is whether they will remain aggregated as biofilms or disperse and return to a motile, planktonic state—is largely governed by exopolysaccharides, as these polymers are a widespread mechanism of bacterial aggregation. There is ample evidence correlating exopolysaccharides with adhesive phenotypes including bacterial aggregation, pellicle and flow-cell biofilm formation, and adherence to surfaces. Classic evidence comes from exopolysaccharide mutants deficient in biofilm formation, which can reveal the role of the polymers in aggregation, but redundant mechanisms of biofilm formation can mask this phenotype. Further evidence stems from overexpression of exopolysaccharide biosynthesis genes, resulting in increased matrix production, enhanced biofilm biomass, and auto-aggregation in liquid culture. Scanning electron microscopy allows for the direct visualization of exopolysaccharides physically connecting cells in biofilms and interacting with other extracellular matrix components (Serra et al. 2013a). The use of optical tweezers to immobilize bacterial cells offers further confirmation that the Pel polysaccharide is critical for cell-to-cell connections in *P. aeruginosa* strain PA14, promoting the retention of daughter cells in a growing aggregate (Colvin et al. 2011). It is interesting to note that while continuous *pel* expression is needed for continued biofilm growth, Pel is not required for maintenance of existing biofilm structure. This is in contrast with the continuous expression of *psl* that is required to maintain existing biofilms. This suggests that the mechanism of Pel-mediated adhesion is more stable than Psl-mediated biofilm adhesion. In conclusion, multiple lines of evidence indicate exopolysaccharides play crucial roles in the aggregation of bacteria into biofilms. The contrasting stability observed between Pel- and Psl-dependent biofilms implies that these exopolysaccharides harbor distinct mechanisms of biofilm adhesion.

Besides cell-to-cell aggregation, the adhesive properties of exopolysaccharides facilitate adherence of bacteria to a variety of biotic and abiotic surfaces. Individual exopolysaccharides differ in their ability to adhere to surfaces. An interesting example of this is Psl, which is important for the ability of *P. aeruginosa* to attach to glass (Colvin et al. 2011; Cooley et al. 2013). In contrast, Pel readily binds mucin and other anionic host polymers (Jennings et al. 2015), but does not attach well to glass (Colvin et al. 2011). The role of Pel in attachment to glass might not be observed because type IV pili—known to mediate surface attachment and twitching motility—may compensate for a lack of Pel (Vasseur et al. 2005; Branda et al. 2005). The significance of exopolysaccharides in bacterial adherence to an abiotic surface depends on the specific type of polysaccharide, underscoring the unique and varied roles that these polymers play in surface attachment.

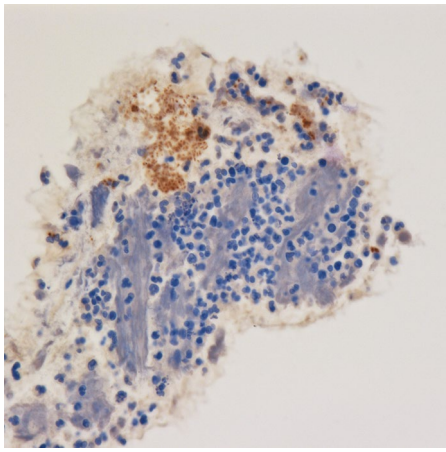

Fig. 1.2 *P. aeruginosa* aggregates actively expressing the Pel polysaccharide in CF sputum. Exopolysaccharides were visualized using immunohistochemistry with Pel-specific antibody (brown), background staining (blue), and neutrophil nuclei (dark blue). The figure displays distinct aggregates along with individual rod-shaped *P. aeruginosa*

Abundant in vitro evidence supports the significance of exopolysaccharides in bacterial aggregation and surface adherence, yet the understanding of their presence and role in vivo remains limited. A notable exception emerged from immunohistochemistry, where specific antibodies revealed that *P. aeruginosa* aggregates in CF sputum produce Pel and Psl (Fig. 1.2) (Jennings et al. 2021). Moreover, aggregate morphology was consistent with a polysaccharide-dependent aggregation mechanism (Jennings et al. 2021). The upregulation of Pel and other biofilm-related genes in a murine pneumonia model further substantiates the discovery of exopolysaccharide production and biofilm formation at infection sites (Fothergill et al. 2014). Thus, a combination of in vitro and in vivo evidence solidly supports the pivotal role of exopolysaccharides in bacterial adherence to surfaces and their aggregation into biofilms.

1.4.2 Biofilm Mechanics: Exopolysaccharides Confer Viscoelastic Properties and Stability to Biofilms

The investigation of biofilm mechanics, which delves into the material properties and responses of biofilms to external forces, is important in the persistence and control of biofilms (Gloag et al. 2020). Biofilms are characterized as viscoelastic materials exhibiting both viscous (resistance to flow) and elastic (adaptable/stretchy) properties. When exposed to mechanical or chemical methods of clearance, the viscoelastic properties of biofilms resist detachment and promote larger and stronger biofilms (Peterson et al. 2015). Mechanical properties of biofilms can be assessed by applying forces such as indentation (compression) or shear stress (horizontal flow or spinning) and measuring the ensuing response or deformation of the biofilm (Fig. 1.3a). When viscoelastic biofilms deform under constant stress, they transition from elastic to viscous behavior; and upon stress removal, they partially recoil, but

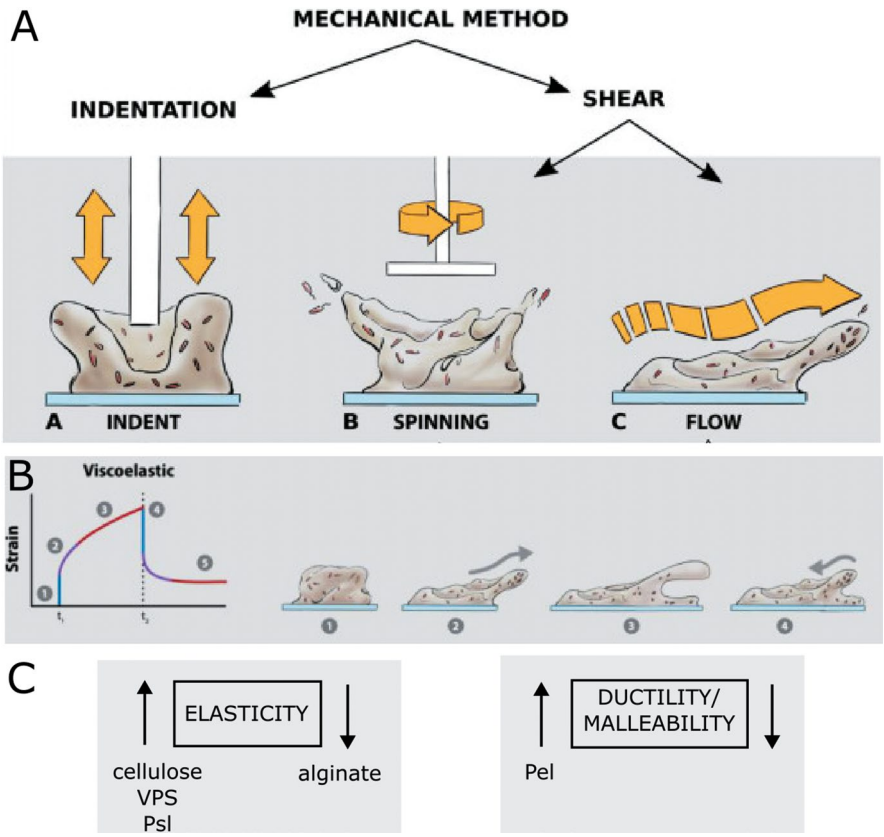

Fig. 1.3 Exopolysaccharides confer viscoelastic properties to biofilms. (**a**) Methods to assess mechanical properties of biofilms include compression, spinning, or flow. Shear force: A force that is applied parallel to a surface. (**b**) Mechanical response of a viscoelastic biofilm to constant stress such as continuous flow or spinning. Stress: The force applied over a unit area (force/area). Strain: Shape change or physical deformation of a material relative to the original dimensions of the material; dimensionless. (**c**) Viscoelastic properties are conferred to biofilms by specific exopolysaccharides. Ductility: Ability of material to deform without breaking under tensile stress. Malleability: Ability of a material to deform without breaking under compressive stress. Figure modified from Gloag et al. (2020), licensed by CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

will never fully return to their original form (Fig. 1.3b). Generally, biofilms cultivated under high flow (shear stress) tend to result in thicker and stronger biofilms. The cohesive nature of biofilms is significantly influenced by exopolysaccharides (Flemming and Wingender 2010), which play a crucial role in determining the viscoelastic properties of biofilms and influence their ability to resist mechanical disruption (Hwang et al. 2014).

Individual exopolysaccharides can confer distinct mechanical properties to biofilms (Fig. 1.3c) (Gloag et al. 2020). In *Vibrio cholera* biofilms, *V*ibrio *P*oly*S*accharide (VPS) increases biofilm elasticity (Yan et al. 2019). In *E. coli*, cellulose confers

cohesion, elasticity, and stability to colony biofilms, compared to biofilms with a proteinaceous curli matrix, which are rigid and brittle (Serra et al. 2013a). The increased elasticity of cellulose biofilms is thought to be due in part to the high water absorption capacity of the polysaccharide (Klemm et al. 2011). Each of the three *P. aeruginosa* exopolysaccharides confers distinct mechanical properties to the biofilm such that Psl increases biofilm elasticity, Pel increases ductility and malleability (Chew et al. 2014; Kovach et al. 2017), and alginate reduces biofilm viscosity and elasticity (Wloka et al. 2005; Gloag et al. 2018). The ratio of mannuronic and guluronic acid in alginate dictates the viscoelastic properties of biofilms, influencing the cough clearance of *P. aeruginosa* from the airways of individuals with CF (Thi et al. 2020). Conceivably, biofilms may alter their exopolysaccharide content, and therefore their mechanical properties, to resist external forces such as variation in flow. Alternatively, the presence of subpopulations of bacteria in biofilms that produce distinct exopolysaccharides could allow the environment to select under stress or changing conditions. Different exopolysaccharides impart unique mechanical properties to biofilms, and the capacity to switch between them may facilitate adaptation and enhance survival (Gloag et al. 2020).

1.4.3 Building and Breaking Biofilms: Exopolysaccharides Shape the Biofilm Lifecycle from Initial Attachment to Dispersal

The formation of biofilms is a controlled, developmental process that is initiated by the reversible attachment of free-swimming, planktonic bacteria to a surface, followed by permanent attachment, microcolony formation and maturation, and finally dispersal to recolonize another environment (O'Toole et al. 2000). The conceptual model of the biofilm was recently expanded to include non-surface attached bacterial aggregates, which are common in shaken cultures and at sites of infection (Kragh et al. 2023; Sauer et al. 2022). This simplified biofilm model includes three stages: (1) aggregation and attachment, (2) growth and accumulation, and (3) disaggregation and detachment (Sauer et al. 2022). Regardless of whether a biofilm is associated with a surface, the biofilm lifecycle allows bacteria to adapt to changes in their environment by switching between sedentary and motile lifestyles—a process that is critical for bacterial survival under changing conditions (Flemming et al. 2023).

Exopolysaccharides play key roles at nearly every stage of the biofilm lifecycle (Fig. 1.4). As an example, at the earliest stages of *P. aeruginosa* biofilm formation, exopolysaccharides facilitate the self-organization of *P. aeruginosa* into microcolonies. During this process, the exploration of a surface is initiated by exopolysaccharide "trails" of Psl deposited from a motile *P. aeruginosa* cell (Zhao et al. 2013). Psl trails attract other *P. aeruginosa* cells, resulting in a positive feedback loop that serves as the founding population for the formation of small aggregates of bacteria

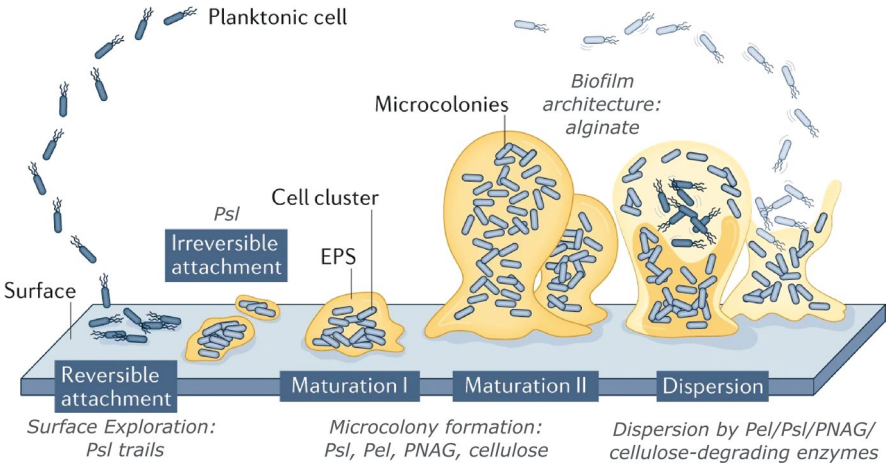

Fig. 1.4 Exopolysaccharides play important roles throughout the lifecycle of a biofilm, from the initial attachment stage to mature aggregate formation and dispersal. Figure modified with permission from Sauer et al. (2022)

referred to as microcolonies. The initial attachment of bacteria to a surface is a loose association, reversible, and mediated by type IV pili or polar flagella (Armbruster and Parsek 2018; Lee et al. 2018). Following the reversible attachment stage, bacteria irreversibly attach to the surface. The irreversible attachment stage coincides with the production of extracellular matrix components including exopolysaccharides. During this stage, cells lie flat against the surface and are resistant to being dislodged. Exopolysaccharides are critical for microcolony formation and maturation, physically connecting cells to other cells and other biofilm matrix components.

The degradation of exopolysaccharides is important for dispersal of bacteria from a biofilm. The increased expression of matrix-degrading enzymes breaks down biofilm exopolysaccharides, releasing biofilm cells and returning them to a single-celled, planktonic state (Rumbaugh and Sauer 2020). Native dispersion refers to the scattering of cells from the biofilm in response to signaling molecules or cues synthesized from biofilm cells, as opposed to environmentally induced cues. The process of native dispersion initiates in a subpopulation of biofilm cells at the center of the biofilm aggregate, generating characteristic voids or cavities at the center of mature microcolonies. There are multiple mechanisms that can mediate native dispersion. At least two of these involve exopolysaccharides, including (1) a reduction in c-di-GMP and (2) an increase in expression of exopolysaccharide-degrading enzymes. More specifically, under low oxygen conditions at the center of the biofilm aggregate, anaerobic denitrification leads to the production of nitric oxide (NO), reducing intracellular c-di-GMP, and exopolysaccharide production (Webb 2009). In addition, NO reacts with superoxide causing cell damage, bacteriophage induction, and lysis of cells at the center of the biofilm (Webb et al. 2003). The lysis of this subpopulation of cells releases matrix-degrading enzymes such as the glycosyl hydrolases PelA, PslG, and dispersin B, which degrade the biofilm matrix

exopolysaccharides Pel, Psl, and PNAG, respectively (Cherny and Sauer 2020; Rumbaugh and Sauer 2020). Interestingly, staphylococci are not known to produce dispersin B or other PNAG-degrading enzymes, but rather rely on the secretion of surfactants known as phenol-soluble modulins that disrupt hydrophobic or hydrophilic interactions between bacterial surface molecules to induce biofilm dispersion (Kaplan et al. 2003; Otto 2014). Thus, exopolysaccharides play key roles not only in building biofilms but also in breaking them down.

1.4.4 Carbohydrate Architects: Influence of Exopolysaccharides on Three-Dimensional Structure and Localization in Biofilms

The biofilm matrix is assembled into intricate three-dimensional structures (Reichhardt and Parsek 2019). Exopolysaccharides play important roles in the spatial organization of the biofilm matrix and distinct biofilm architecture. Exopolysaccharides contribute to a highly ordered biofilm matrix that is dynamic in space and time (Flemming et al. 2023). The complexity of the biofilm matrix can be likened to a rudimentary form of cell differentiation (Stoodley et al. 2002). One manifestation of this complexity is observed in the localization of exopolysaccharides around individual bacterial cells and within the biofilm. For instance, Psl is anchored to the surface of bacteria in a helical pattern that is thought to promote cell–cell and cell–surface interactions with the polysaccharide (Ma et al. 2009). Within *P. aeruginosa* biofilms, Pel and Psl are localized to different regions. Psl accumulates in the periphery of the biofilm aggregate overtime, while the interior of mature aggregates is largely void of the polysaccharide (Ma et al. 2009). In contrast, Pel localizes to the interior of the aggregate near the base of the biofilm, but in strains that lack Psl, Pel can compensate and localize to the periphery (Fig. 1.5) (Jennings et al. 2015), suggesting that the regulatory pathways of these polysaccharides are intertwined by a yet-to-be-determined mechanism. The positioning of Pel and Psl in biofilms provides insight into their biological roles, suggesting that the polysaccharides serve both unique and overlapping functions in the *P. aeruginosa* biofilm matrix.

In *E. coli*, nutrient gradients in colony biofilms result in the differentiation of cells into a two-layer architecture (Fig. 1.6) (Serra et al. 2013b). The lower region of the biofilm near the nutrient-agar source contains rod-shaped, dividing cells in post-exponential growth. The top layer of the biofilm is characterized by small ovoid cells with a stationary-phase physiology due to nutrient limitations. Biofilm matrix components are produced in discrete zones of *E. coli* biofilms, and the composition of the matrix appears to correlate with the metabolic state of the cells within the region (Hobley et al. 2015). In the upper portion of the colony biofilm where nutrients are limited, ovoid *E. coli* cells produce the biofilm matrix components cellulose and proteinaceous curli fibers (Serra et al. 2013a). Cellulose is a linear

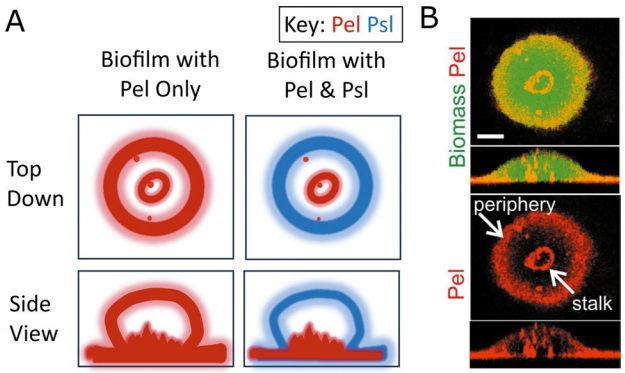

Fig. 1.5 Localization of Pel and Psl in biofilms depends on the biofilm matrix composition. (**a**) In biofilms without Psl, Pel localizes to the periphery and stalk of the biofilm aggregates. In biofilms with both polysaccharides, Psl localizes to the periphery, and Pel resides in the stalk. (**b**) Confocal microscopy of biofilm overexpressing Pel (PAO1 Δ*wspF* Δ*psl* P_{BAD} *pel*). Pel-specific WFL lectin (red), *P. aeruginosa* biomass (green). Adapted with permission from Jennings et al. (2015)

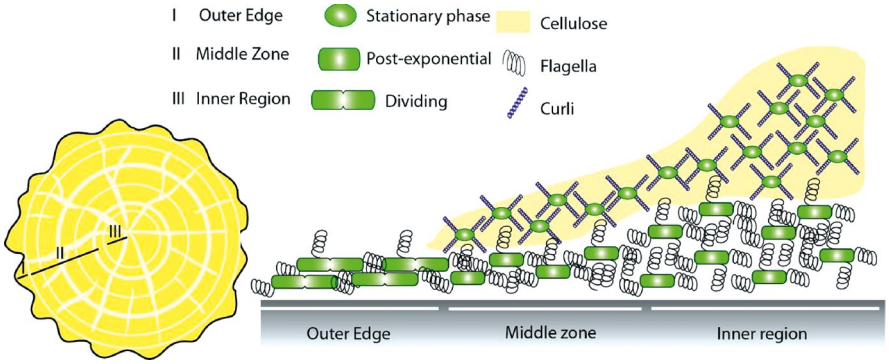

Fig. 1.6 Localization of cellulose and other biofilm matrix components in an *E. coli* colony biofilm. The figure used with permission from Hobley et al. (2015), licensed by CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

homopolymer of β-1,4-linked glucose that may contain phosphoethanolamine modifications produced by *E. coli* and other bacteria and plants (Wang et al. 2019; Thongsomboon et al. 2018). In contrast, rod-shaped cells at the bottom and outer edge of the colony near the nutrient agar contain flagellar filaments. The demarcation of the cells and exopolysaccharide components in the biofilm suggests that under nutrient-limiting conditions *E. coli* produces biofilm matrix components like cellulose, but when conditions are favorable for growth near the nutrient agar, *E. coli* produces flagella. Cellulose can "wheel lock" flagellar rotation leading to a decrease in the expression of motility genes (Zorraquino et al. 2013), which is one example of how exopolysaccharide production is tightly linked to inhibition of motility. The differentiation of bacterial cells in biofilms into two distinct physiological cell types

that are optimized for (1) maintenance/survival or (2) growth may serve as a division of labor or bet-hedging strategy to protect the biofilm bacteria from stresses or changing environmental conditions.

One common way that exopolysaccharides contribute to the three-dimensional architecture of colony biofilms is through the buckling of the colony into ridges forming a complex network of wrinkles. Wrinkled or rugose colony morphology (sometimes called rugose small colony variants, RSCVs) has been observed in many biofilm-forming organisms including *P. aeruginosa*, *P. fluorescens, V. cholerae, E. coli*, and *B. subtilis* and is often correlated with increased intracellular concentrations of c-di-GMP, enhanced exopolysaccharide production, and hyper-biofilm formation (Kirisits et al. 2005; Aguilar et al. 2007; Lim et al. 2006; Serra et al. 2013a; Xu et al. 2021). In *P. fluorescens*, rugose colonies are referred to as wrinkly spreaders, which evolve for the competition of oxygen in pellicle biofilms that form at the air–liquid interface of standing cultures (Koza et al. 2011). In these static cultures, bacterial growth is oxygen limited, and high oxygen concentrations at the air–liquid interface favor the formation of the wrinkly spreader phenotype. It is thought that wrinkling increases the surface area of the biofilm, enhancing access to oxygen and other nutrients, which could be beneficial under anoxic conditions. Rugose colony variants are of potential clinical significance because they are frequently isolated from chronic infections and resistant to treatment (Gloag et al. 2019; Evans 2015). The role of exopolysaccharides in the architecture of rugose colony biofilms is a common trait across diverse bacteria, facilitating adaptation to oxygen limitation and other environmental stressors.

Antibodies targeting the Psl exopolysaccharide in *P. aeruginosa* biofilms reveal a highly organized arrangement of the polysaccharide within the biofilm matrix. Human monoclonal antibodies (mAbs) against Psl were discovered through phenotypic screening (Digiandomenico et al. 2012). Epitope mapping of these anti-Psl antibodies onto a panel of synthetic oligosaccharides revealed that the antibodies recognize three distinct epitopes within the Psl polysaccharide, referred to as class I, II, and III (Li et al. 2013). The different classes of mAbs showed differential ability to inhibit biofilm formation and aggregation. Intriguingly, upon application of fluorescently labeled anti-Psl mAbs to both static and flow-cell biofilms, a stratified staining pattern emerged. Class I mAb predominantly stained the upper layer or periphery of the biofilm aggregate, while class II mAb targeted the layer beneath, and class III mAb stained the region near the biofilm base or center of the aggregate (Fig. 1.7) (Ray et al. 2017). The authors propose that the stratification of Psl epitopes in biofilms may arise from distinct forms of Psl, or reflect phenotypically diverse subpopulations within the biofilm structure.

Analysis of the chemical structure and localization of anti-Psl mAb epitopes provides valuable insights into the distribution of potentially distinct forms of Psl in biofilms. Psl, characterized as a branched pentasaccharide repeat of mannose, rhamnose, and glucose (Byrd et al. 2009), exhibits variations in epitopes based on location within the biofilm. Near the base of the biofilm, Class III mAbs exhibited the strongest binding affinity to synthetic oligosaccharides with a terminal glucose residue. Given that low-molecular-weight Psl contains more terminal glucose sugars

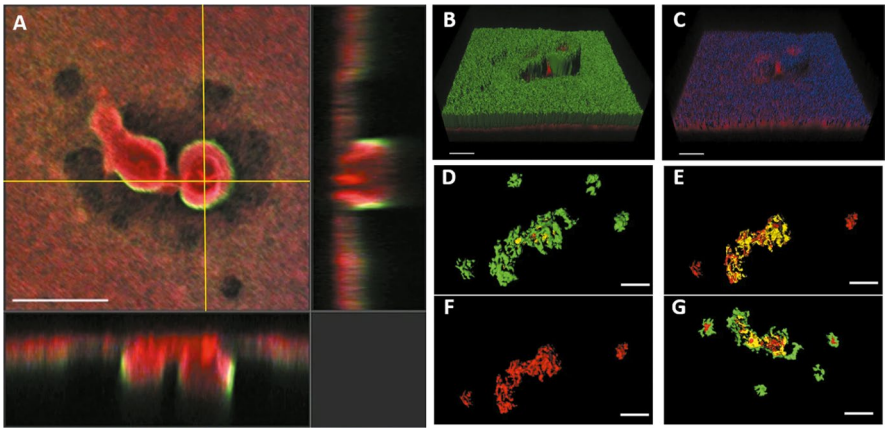

Fig. 1.7 Stratification of Psl epitopes suggests different forms of Psl in biofilms. Flow-cell (**a**–**c**) and static (**d**–**g**) biofilms stained with anti-Psl mAbs: class I (green), class II (blue **a**–**c** or yellow **d**–**g**), and class III (red). Image processing: (**c**, **e**) green layer removed, (**f**) green and yellow layers removed. Point of view: (**d**) from the top, (**g**) from the bottom. Scale bars (**a**) 100 μm, (**b**, **c**) 40 μm, and (**d**–**g**) 150 μm. Figure used with permission from Ray et al. (2017), licensed by CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)

than its higher-molecular-weight counterpart, this observation suggests that the Psl near the base of the biofilm may undergo processing or degradation resulting in smaller length polymers. This is interesting given that Psl has a high- and low-molecular-weight form, but it is not clear how they are derived or distributed in biofilms. Class II mAbs, which stained between the other antibodies in the biofilm, reacted with each of the synthetic Psl oligosaccharides tested suggesting that the antibodies bind the Psl "backbone" and do not require the branched mannose sugar. Unexpectantly, class I mAbs, which stained the top layer of the biofilm (or periphery of the aggregate), did not react with any of the synthetic oligos tested. This is surprising given that all classes of mAb reacted with Psl in planktonic cultures. Several plausible scenarios could explain this biofilm-dependent phenomenon, including the class I mAb recognizing Psl that is: (1) folded upon itself, (2) bound to other matrix components, such as the CdrA adhesin, or (3) chemically modified by a biofilm matrix component. This is an interesting area of research that warrants further study. The ability to discern between different forms of Psl within a biofilm not only unveils the intricacies of the biofilm matrix, but also raises the possibility that other exopolysaccharides may manifest distinct forms within biofilms as well.

1.4.5 *Resistant by Design: Exopolysaccharide-Mediated Defense Against Antimicrobials*

One of the defining traits of biofilms is their enhanced survival upon exposure to antimicrobials such as antibiotics, disinfectants, and toxic metals. Biofilms can tolerate antibiotics at concentrations 100–1000 times higher than the minimum inhibitory concentration (MIC) for planktonic cells. This remarkable contrast in antibiotic susceptibility is, in part, attributed to exopolysaccharides. Evidence indicates that the heightened expression of biofilm matrix components, including alginate, Pel, and Psl resulting from mutations frequently identified in clinical isolates, shields *P. aeruginosa* aggregates cultivated in gels from the effects of antibiotic treatment. Aggregates formed by Δ*mucA* mutants, characterized by the overproduction of alginate, and Δ*wspF* mutants, which exhibit elevated production of Pel and Psl, demonstrated a 30–50 fold increase in tolerance to tobramycin compared to wild-type aggregates (Goltermann and Tolker-Nielsen 2017). Notably, the restoration of antibiotic susceptibility occurred upon the deletion of exopolysaccharide biosynthesis genes in isogenic strains, underscoring the specificity of the observed phenotypes to the exopolysaccharides.

The tolerance of biofilms to antimicrobials is mediated by exopolysaccharides through two main mechanisms:

1. *Direct interactions with antimicrobials*: These involve processes such as sequestration, binding, or inactivation/degradation of antimicrobials by exopolysaccharides. These interactions reduce the effective concentration of the antimicrobial to sublethal levels, contributing to the biofilm's ability to withstand treatment.
2. *Indirect mechanisms*: These arise from exopolysaccharide-mediated nutrient gradients within biofilms that generate phenotypic heterogeneity or the slow growth rate (or no growth) of a small subpopulation of biofilm cells called "persisters" that are tolerant to high concentrations of antimicrobials.

As a consequence of these mechanisms, the effective impact of antimicrobials is diminished, either due to the limited penetration into biofilm structures or the reduced metabolic activity of certain cells. It's important to note that the term "antimicrobial tolerance" in this context refers to a non-heritable trait or transient phenotype. Unlike antimicrobial resistance, which is a genetically encoded and heritable characteristic acquired through mechanisms like mutation or horizontal gene transfer, antimicrobial tolerance typically diminishes when biofilm cells disperse.

Biofilms can develop tolerance to aminoglycosides through direct interactions of the antibiotics with exopolysaccharides. Aminoglycosides, known for their bactericidal nature targeting the ribosomes of both Gram-negative and Gram-positive bacteria, disrupt protein synthesis. In *P. aeruginosa,* anionic cyclic glucans protect biofilms from aminoglycoside antibiotics through direct binding. The *ndvB* locus, which is required for the synthesis of periplasmic cyclic-β-(1,3)-glucans, was identified in a screen of transposon mutants deficient in biofilm-mediated tolerance to antibiotics (Mah et al. 2003; Sadovskaya et al. 2010). Disruption of the *ndvB* gene

increased antibiotic sensitivity to tobramycin and other antibiotics in multiple biofilm models (Mah et al. 2003). Hydrophobic interaction chromatography indicated that purified glucans and tobramycin interact in vitro. These findings demonstrate that periplasmic glucans actively sequester antibiotics, diminishing the diffusion of these molecules to their cytoplasmic site of action. In this antibiotic tolerance mechanism, the negative charge of the cyclic glucans is consistent with their ability to bind positively charged aminoglycosides.

Alginate also contributes to antibiotic tolerance by directly binding aminoglycosides. In biofilms formed by *mucA* mucoid strains of *P. aeruginosa* that exhibit overproduction of alginate, a marked increase in tolerance to tobramycin is observed, compared to non-mucoid strains (Hentzer et al. 2001). This biofilm-specific effect was validated using two independent biofilm-culturing techniques, namely, flow-cell and rotating-disk reactor models. Alginate directly bound to tobramycin reducing the diffusion of the antibiotic, leading to a reduction in the zone of growth inhibition (Nichols et al. 1988). Other studies demonstrated that alginate not only hindered the diffusion but also completely blocked the entry of tobramycin and gentamycin into biofilms, although it had no such effect on carbenicillin (Hatch and Schiller 1998). Treatment of biofilms with alginate lyase, an enzyme that catalyzes the cleavage of the glycosidic linkages in alginate, restored diffusion and sensitivity to aminoglycoside antibiotics (Hatch and Schiller 1998; Alkawash et al. 2006). Collectively, these findings suggest that the negative charge on alginate likely facilitates its binding to cationic aminoglycoside antibiotics, impeding their penetration and contributing significantly to antimicrobial tolerance within biofilms.

The biofilm matrix, along with its embedded exopolysaccharides, acts as a physical impediment that restricts the diffusion of certain antibiotics, either through physical obstruction or molecular binding (De Beer et al. 1997). Alginate and cyclic glucans are examples of specific polysaccharides that can reduce the diffusion of aminoglycoside antibiotics by direct binding. In general, the penetration of charged antibiotics like aminoglycosides into biofilms occurs at a slower rate compared to fluoroquinolones like ciprofloxacin that are neutral at physiologically relevant pH and readily penetrate biofilms (Walters et al. 2003; Tseng et al. 2013; Jefferson et al. 2005). Birjiniuk et al. utilized single-particle tracking microscopy with functionalized beads to assess the transport of particles with varying charges into *E. coli* flow-cell biofilms (Birjiniuk et al. 2014). Their findings indicated that neutral PEGylated particles exhibited faster penetration through biofilms compared to positively charged aminated beads or negatively charged carboxylated beads. They went on to show that (1) charge density increased in the deeper layers of the biofilm, (2) larger particles became entrapped within the biofilm, and (3) the biofilm contained fluid-filled channels, highlighting the dependence of diffusion in biofilms on factors such as charge, particle size, and channeling. Others showed that charge interactions were important for transport of nanoparticles into young, but not mature biofilms (Rodríguez-Suárez et al. 2023).

Jacobs et al. observed that alginate polymers cross-linked with calcium slowed the penetration of antibiotics into *P. aeruginosa* colony biofilms, but the reduced transport could not be exclusively attributed to charge-based interactions, as there

were limited differences in the ability of negatively charged fluorescein or positively charged rhodamine B to penetrate the biofilms (Jacobs et al. 2022). These findings emphasize that charge-based interactions can limit antibiotic penetration into the biofilm matrix and protect the community from antibiotic-induced eradication, but they are not the sole factors behind enhanced antimicrobial tolerance observed in biofilms. Additionally, multifaceted mechanisms contributing to antibiotic tolerance within biofilms require further exploration.

Cationic polysaccharides play a crucial role in shielding bacteria from antimicrobials through repulsion and sequestration mechanisms. One notable illustration is the Pel polysaccharide, which enhances the antimicrobial tolerance of *P. aeruginosa* biofilms to aminoglycosides, like tobramycin and gentamicin (Khan et al. 2010; Colvin et al. 2011). This protective effect is specific to Pel and is not observed with Psl or the neutral antibiotic ciprofloxacin. Instead, it hinges on Pel's ability to engage in ionic interactions with extracellular DNA (eDNA) (Jennings et al. 2021). Biofilms rich in Pel effectively sequester eDNA into the matrix, thereby augmenting tolerance to tobramycin. This finding is consistent with previous studies indicating that eDNA contributes to antibiotic tolerance/resistance by (1) directly binding to the antibiotic (Chiang et al. 2013) and (2) establishing a cation-limited environment that triggers the induction of the cationic antimicrobial peptide resistance operon *pmr* (Wilton et al. 2016). Pel-eDNA interactions hold potential clinical significance since eDNA is an important biofilm matrix component that is not only prevalent in mature biofilm aggregates but also at sites of human infection such as the CF airways. Both direct and indirect interactions of exopolysaccharides with aminoglycosides impact the pathogen's tolerance to antibiotics, and ultimately, the persistence of infections and treatment outcomes.

Exopolysaccharides within the biofilm matrix exhibit both reactive and dynamic functions, capable of inactivating antimicrobials and adjusting their matrix constituents in response to environmental changes. A noteworthy example of a reactive exopolysaccharide is alginate, which was shown to protect *P. aeruginosa* from chlorine disinfectants (Hodges and Gordon 1991). The absence of alginate production renders *P. aeruginosa* more susceptible to chlorine (Xue et al. 2013). The tolerance mechanism relies on the acetyl substituents on alginate, which react with free chlorine to neutralize the oxidizing disinfectant (Flemming et al. 2023). Beyond neutralizing antimicrobials, biofilms can modify the exopolysaccharides within their extracellular matrix to adapt to new environmental threats. *P. putida*, for instance, significantly increases exopolysaccharide production in response to the organic pollutant toluene. This results in a biofilm matrix with an elevated number of carboxyl groups, enhancing sorption capacity and the ability to bind metal cations (Schmitt et al. 1995). Another mechanism by which exopolysaccharides facilitate the adaptation of the biofilm to changing conditions is by holding bacteria in close physical proximity, creating an environment conducive to genetic exchange (Sørensen et al. 2005; Abe et al. 2020). Because of this, there is an increased opportunity in biofilms to develop antibiotic resistance through the horizontal gene transfer of efflux pumps or other resistance genes. In conclusion, the biofilm matrix has evolved over extended periods of time to enhance the odds of survival under inhospitable or

changing environmental conditions. Exopolysaccharides are an important part of this process.

1.5 Structure and Function of Common Bacterial Exopolysaccharides

1.5.1 Alginate: A Key Player in CF Pathogenesis and Biofilm Persistence

Pseudomonas aeruginosa, an opportunistic pathogen and a model organism for biofilm studies, stands as a significant contributor to both acute and chronic biofilm infections. This is particularly true for individuals with the genetic disorder CF, those with ventilator-associated pneumonia, chronic wounds, or hospital-acquired infections (Gibson et al. 2003; Ramírez-Estrada et al. 2016; Mulcahy et al. 2014). Strains of *P. aeruginosa* are increasingly resistant to multiple classes of antibiotics; and currently, there is no approved vaccine for human use (Priebe and Goldberg 2014; Breidenstein et al. 2011). This dearth of viable treatment options intensifies the challenge of addressing infections caused by this formidable pathogen. *P. aeruginosa* can produce multiple exopolysaccharides that have been implicated in biofilm formation, including alginate, Psl, and Pel (Franklin et al. 2011).

Alginate, initially identified in brown seaweed, was subsequently found in *P. aeruginosa* and various other Gram-negative bacterial species. While the structure of alginate may vary among genera, it consistently represents a high-molecular-weight anionic polysaccharide composed of non-repeating subunits of β-1,4 linked D-mannuronic and L-guluronic acid residues (Evans and Linker 1973). Acetylation of alginate can occur selectively at the O_2 and/or O_3 positions of the β-D mannuronic acid residues, resulting in its somewhat random conformation and an increased water retention capacity (Hay et al. 2010).

Alginate is encoded by a 12-gene operon (PA3540-PA3551 in PAO1). The synthesis of alginate involves the binding of the signaling molecule c-di-GMP to the cytoplasmic membrane protein Alg44 (Franklin et al. 2011). Initially, alginate is formed as a homopolymer of D-mannuronic acid residues (Franklin et al. 2011). Subsequently, in the periplasm, the polymer undergoes selective *O*-acetylation and epimerization, facilitated by the AlgG epimerase, which converts the D-mannuronic acid residues to L-guluronic acid. Finally, the fully developed polymer is secreted through AlgE, a highly electropositive pore spanning the outer membrane.

Alginate plays a pivotal role in shaping biofilm architecture and contributing to resistance properties. The overproduction of alginate leads to a mucoid phenotype and changes in the three-dimensional structure of the biofilm (Stapper et al. 2004; Hentzer et al. 2001). Mucoid strains tend to form thicker biofilms compared to the more densely packed biofilms of non-mucoid strains. The structural integrity of mucoid biofilms is specifically attributed to *O*-acetylated alginate (Nivens et al.

2001). In *Pseudomonas fluorescens*, mucoid strains exhibit increased adherence to plant roots, underscoring the significance of alginate in attachment (Bianciotto et al. 2001). While alginate is not a strict requirement for biofilm formation, studies have demonstrated its role in providing enhanced water retention (Mann and Wozniak 2012). In *P. fluorescens*, an *algU* mutant deficient in alginate production, is more susceptible to desiccation and osmotic stress (Schnider-Keel et al. 2001). Thus, alginate contributes to both biofilm architecture and desiccation resistance.

Alginate serves as a critical virulence factor with a significant role in airway infections affecting patients with the genetic disorder CF (Pedersen et al. 1992). CF is caused by mutations in the CF transmembrane conductance regulator (CFTR). Defects in this chloride ion channel lead to the thickening of the mucus layer in the CF airways, diminishing mucociliary clearance of bacterial pathogens (Boucher 2004). Despite repeated antibiotic treatments, bacterial infections persist in the CF airways, contributing to the progressive loss of lung function, and elevated morbidity and mortality rates (Govan and Deretic 1996; Lyczak et al. 2002). The majority of individuals with CF, up to 80%, eventually become colonized with *P. aeruginosa* (Moreau-Marquis et al. 2008). The lungs of CF patients are initially colonized by non-mucoid *P. aeruginosa* strains, but by the time the lung infection has developed into a chronic established infection, the predominant pathogen in the lungs is a mucoid strain of *P. aeruginosa* that secretes copious amounts of alginate (Ramsey and Wozniak 2005). Infections with mucoid strains correlate with reduced lung function, poorer clinical outcomes, and an increased risk of death (Pedersen et al. 1992). Significant research attention has been devoted to alginate due to its correlation with the transition from non-mucoid to mucoid strains in the lungs of CF patients, which is associated with a deteriorating clinical prognosis (Govan and Deretic 1996).

Alginate hinders the innate immune system's ability to eliminate *P. aeruginosa* from the CF respiratory tract through multiple mechanisms (Ramsey and Wozniak 2005). Following a bacterial infection, immune cells are recruited to the site and act as the first line of defense against invading pathogens. To subvert the immune system, alginate (1) scavenges reactive oxygen species, such as hypochlorite released from activated macrophages (Learn et al. 1987; Simpson et al. 1989), (2) serves as a barrier, protecting against phagocytic clearance (Govan and Deretic, 1996a), and (3) reduces the chemotaxis of polymorphonuclear leukocytes (PMN) in the CF airways (Pedersen et al. 1990). Additionally, *O*-acetylation of alginate is crucial for *P. aeruginosa* resistance to opsonic phagocytosis (Pier et al. 2001). Although antibodies to alginate are present in individuals with chronic CF lung infections, these antibodies are not protective and fail to mediate opsonic killing of *P. aeruginosa* in vitro (Ramsey and Wozniak 2005). Consequently, once a chronic lung infection is established with a mucoid strain, the infection can be extremely difficult to eradicate because alginate enhances resistance to antimicrobials and contributes to the evasion of the host immune response through diverse processes.

1.5.2 *Psl: Master Architect of Biofilm Structure, Built to Evade Immune Cells and Predators*

Although alginate influences biofilm architecture and resistivity, it is not indispensable for biofilm formation, as alginate-deficient strains still demonstrate the ability to form biofilms. Biofilms from the non-mucoid strains PAO1 and PA14, commonly isolated from environmental samples and implicated in the initial stages of CF lung infections, produce little to no detectable alginate in vitro (Wozniak et al. 2003). This observation, coupled with the sequencing of the *P. aeruginosa* genome, led to the identification of two additional exopolysaccharides crucial for biofilm formation in non-mucoid Pseudomonads, namely Psl and Pel. The *p*olysaccharide *s*ynthesis *l*ocus (*psl*) was independently identified by three research groups through high-throughput screening of transposon mutants deficient in wrinkly colony morphology and/or the targeted generation of deletion mutants of genes homologous to exopolysaccharide biosynthetic functions (Jackson et al. 2004; Friedman and Kolter 2004b; Matsukawa and Greenberg 2004).

The Psl structure consists of a branched, repeating pentasaccharide composed of D-mannose, D-glucose, and L-rhamnose in a 3:1:1 ratio (Byrd et al. 2009). Psl carries an overall neutral charge. Size exclusion chromatography reveals two forms of the Psl polysaccharide: a high-molecular-weight cell-associated form (>10 kDa) and a low-molecular-weight secreted form (3–6 kDa) (Byrd et al. 2009). The *psl* operon comprises 15 genes, with eleven genes (*pslACDEFGHIJKL*) being essential for both Psl production and surface attachment (Byrd et al. 2009).

Psl synthesis is induced by elevated levels of c-di-GMP, an intracellular signaling molecule associated with various cellular processes, including biofilm formation and motility (Mann and Wozniak 2012). Psl stimulates two diguanylate cyclases to produce c-di-GMP, thereby promoting biofilm formation in a positive feedback loop (Irie et al. 2012). While Psl is constitutively expressed in planktonic cells (Overhage et al. 2005), its transcription is positively regulated by the σ-factor RpoS and negatively controlled by the post-transcriptional regulator RsmA (Irie et al. 2010).

Polysaccharide biosynthesis typically commences with sugar nucleotide precursors, which can undergo modifications or be directly integrated into the polysaccharide chain. For Psl synthesis, the activated sugars required are GDP-mannose, UDP-D-glucose, and dTDP-L-rhamnose (Byrd et al. 2009). In the *psl* operon, only PslB is involved in sugar nucleotide precursor production, while other enzymes responsible for precursor production are encoded by genes in alternative polysaccharide biosynthesis pathways. Interestingly, PslB is not essential for polysaccharide production because its function is shared with WbpW, an enzyme involved in the synthesis of both Psl and A-band lipopolysaccharide (LPS) (Byrd et al. 2009). The biosynthesis of Psl follows a Wzy-dependent pathway, wherein the mannose-rhamnose-glucose (3:1:1) pentasaccharide repeat is assembled in the cytoplasm. Subsequently, this repeat is loaded onto an isoprenoid lipid carrier, and flipped into the periplasm by the Wzx flippase PslK (Gheorghita et al. 2023). Within the

periplasm, the Wzy glycosyltransferase PslJ adds the next pentasaccharide repeat onto the growing chain to polymerize Psl (Whitfield et al. 2020a). The resulting polymer is then transported across the periplasm and into the extracellular space by PslE and PslD (Franklin et al. 2011).

Non-mucoid strains of *P. aeruginosa* can utilize Psl or Pel as the primary structural scaffold for biofilm development (Colvin et al. 2012). Although it is now appreciated that mucoid strains produce Psl, albeit at a lower level than non-mucoid strains (Jones and Wozniak 2017). The transcription factor AmrZ plays a direct role in repressing Psl synthesis while inducing alginate (Jones et al. 2013). Polysaccharide production varies significantly between strains. For instance, Psl is essential for biofilm formation in PAO1 (Ryder et al. 2007), but not required in PA14. The *psl* operon was found in 94% of clinical isolates of *P. aeruginosa* ($n = 913$), and anti-Psl antibody binding confirmed Psl expression in the diverse isolates (Tabor et al. 2018). The *psl* operon is also conserved in several other Pseudomonads including *P. fluorescens Pf5* and B728a, *P. syringae* strains 1448A, DC3000, and *Pseudomonas mendocina* ymp (Mann and Wozniak 2012). These findings indicate that Psl is encoded by many but not all strains of pseudomonads.

Psl plays a crucial role in the initial attachment of bacteria to both abiotic and biotic surfaces, such as epithelial cells and mucin-coated surfaces, and contributes to the structural stability and architecture of mature biofilms (Ma et al. 2006; Mann and Wozniak 2012; Overhage et al. 2005). Direct visualization of Psl during biofilm development reveals its anchoring to cells in a helical pattern during the initial attachment phase of biofilm formation, and that dissociation of Psl from the cell surface disrupts the biofilm (Ma et al. 2009). In the early stages of biofilm formation, Psl is evenly distributed throughout small microcolonies. As the biofilm matures and microcolonies grow, Psl accumulates on the periphery of the aggregate, creating a central cavity devoid of Psl but filled with planktonic cells and eDNA (Ma et al. 2009). Overall, evidence suggests that Psl serves as a key scaffolding component in many *Pseudomonas* strains, crucial for the initial attachment of cells to surfaces and the architectural development of mature biofilms.

Interactions between Psl and the extracellular matrix protein CdrA play an important role in promoting biofilm formation and providing protection from proteolysis. CdrA is a biofilm adhesin that encodes carbohydrate-dependent hemagglutination domains predicted to engage with sugars within the biofilm or host extracellular matrix. Overexpression of *cdrA* promotes biofilm formation and Psl-dependent aggregation in liquid culture that can be disrupted by the addition of the Psl sugar-monomer mannose, suggesting that CdrA and Psl interact. Co-immunoprecipitation of CdrA and Psl from culture supernatant provides additional evidence of a specific connection (Borlee et al. 2010). The direct binding of Psl to CdrA serves to safeguard the matrix protein from both self-produced and exogenous proteolysis, thereby enhancing biofilm integrity (Reichhardt et al. 2018). Interestingly, CdrA also binds Pel, showcasing the adhesin's capability to interact with diverse exopolysaccharides (Reichhardt et al. 2020). These findings underscore the presence of distinct adhesion mechanisms in the *P. aeruginosa* biofilm

matrix, including protein adhesins and charge-based ionic interactions, which likely contribute to the redundancy observed in biofilm formation.

Psl plays important roles in infection pathogenesis, from attachment to host surfaces to evading the immune system. *P. aeruginosa* isolates from children with CF who did not respond to antibiotic therapy exhibited increased anti-Psl antibody binding in in vitro biofilms, suggesting that Psl may be a factor in the failure of antibiotics to eradicate *P. aeruginosa* from the CF airways (Morris et al. 2021). For an infection to initiate in the airways, *P. aeruginosa* must first attach to airway epithelial cells and interact with mucin secreted into the overlying mucous layers. Studies show that Psl is essential for adherence to airway epithelial cells and mucin-coated surfaces (Ryder et al. 2007). Psl facilitates binding to host tissues and indirectly stimulates flagellin-mediated proinflammatory signaling (Byrd et al. 2010). Evidence suggests Psl provides protection from host defenses, as Psl-deletion mutants were more easily killed by macrophages and neutrophils than wild-type cells due to a reduced oxidative response (Mishra et al. 2012). Both Pel and Psl play key roles in the tolerance to oxidative stress agents, including UVA radiation, sodium hypochlorite, and hydrogen peroxide in *P. aeruginosa* biofilms (Grossich et al. 2023). In summary, Psl promotes the initiation and persistence of infection by facilitating attachment to the host and resistance to immune cell clearance.

Psl plays a significant role in influencing predator–prey interactions. *P. aeruginosa* hinders the motility of the bacterivore nematode *Caenorhabditis elegans* in a manner that is dependent on Psl. This interference leads to a reduction in the velocity of *C. elegans*, causing the nematode to become entrapped in the biofilm matrix to its own detriment (Chan et al. 2021). Through this mechanism, Psl effectively reduces predation by *C. elegans* and promotes *P. aeruginosa* survival. In addition to impeding a predator's motility, both Pel and Psl exopolysaccharides serve to prevent the detection of *P. aeruginosa* by nematode predators (Li et al. 2022). Autoinducers, which are quorum-sensing signals released by bacteria to facilitate bacterial communication, can be sensed by the bacterivorous nematode *C. elegans*' olfactory receptor as chemoattractants, posing a risk of predation to the bacteria. To conceal detection, *P. aeruginosa* entraps the autoinducers within its Pel and Psl extracellular matrix, cleverly preventing *C. elegans* from detecting the chemoattractant. This strategy helps protect the bacteria's communication from interception by predators. In summary, biofilm exopolysaccharides can interfere with both a predator's motility and its ability to detect prey, ultimately reducing predation and promoting the survival of *P. aeruginosa.*

1.5.3 *Pel: Orchestrating Biofilm Structure and Survival Tactics at Infection Sites*

The *pel* operon was initially discovered through a screen of transposon mutants deficient in pellicle formation (Friedman and Kolter 2004a). Pellicles are biofilms that form at the air–liquid interface of standing cultures. The *pel* operon (named for *pel*licle) is composed of seven genes, all of which are required for pellicle formation in *P. aeruginosa* PA14. More recently, the presence of the *pel* operon was detected in a variety of Gram-negative and Gram-positive bacteria, suggesting that Pel production may be widespread among diverse bacteria (Whitfield et al. 2020b; Whitfield and Howell 2021).

Pel exists in two forms: a high-molecular-weight cell-associated form and a low-molecular-weight non-cell-associated form. Size exclusion chromatography revealed that the non-cell-associated form, found in culture supernatant, had a size of 0.5 kDa compared to dextran standards. High-molecular weight Pel, isolated by extraction with EDTA and heat, eluted in the void volume of the size exclusion column, indicating a size larger than 80 kDa (Jennings et al. 2015). Despite Pel's importance in biofilm formation, its structure was not determined for over a decade after its initial discovery. Initially thought to be a glucose-rich polysaccharide (Friedman and Kolter 2004a), it was later determined that the glucose was attributable to cyclic glucans and not the pel locus (Sadovskaya et al. 2010).

The first clue into Pel's structure emerged from a screen of antibodies to other polysaccharides. Antibodies targeting amino-sugar polymers, including chitosan (β-1,4 *N*-acetylglucosamine) and PNAG (β-1,6 *N*-acetylglucosamine), reacted with culture supernatant from a Pel overexpression strain, but not a deletion mutant (Jennings et al. 2015). Furthermore, ion exchange chromatography demonstrated that Pel bound to a cation exchange column and eluted at a pH of 6.7–6.9, indicating that the polymer was cationic and positively charged below this pH, which is consistent with an amino-sugar polymer. The analysis of polysaccharide sugar composition necessitates breaking glycosidic linkages before sugar monomers can be detected using gas chromatography and mass spectrometry (GC/MS). After optimizing conditions for glycosidic bond cleavage to detect amino sugars, the primary sugar component of Pel was identified as *N*-acetylgalactosamine (Jennings et al. 2015). GC/MS linkage analysis determined the primary glycosidic linkage of Pel to be a 1–4 linkage. Solid-state NMR demonstrated that 50% of Pel sugars were deacetylated, giving the polysaccharide its positive charge (Jennings et al. 2021). Partial hydrolysis of Pel with the glycoside hydrolase $PelA_h$ to increase polymer solubility, indicated that Pel consists of a dimeric repeat of α-1,4-linked galactosamine and *N*-acetylgalactosamine (Le Mauff et al. 2022). Overall, the results indicate that Pel is a polycationic, amino sugar polymer, not previously detected in *P. aeruginosa* or other bacteria. It is interesting to note that *P. aeruginosa* produces three exopolysaccharides each with a distinct charge (anionic alginate, neutral Psl, and cationic Pel).

Pel biosynthesis follows a synthase-dependent mechanism, similar to alginate (Gheorghita et al. 2023). The *pel* operon lacks genes predicted for sugar precursor production, suggesting the involvement of enzymes from other pathways. The regulation of polymer synthesis is governed by the c-di-GMP binding protein PelD (Whitney et al. 2012). The *pel* operon contains a single cytoplasmic glycosyltransferase PelF that binds the sugar nucleotide precursor UDP-GalNAc and catalyzes polymerization of an *N*-acetylgalactosamine (GalNAc) homopolymer prior to translocation of the polysaccharide to the periplasm by PelG. In the periplasm, the polymer undergoes modification by the deacetylase domain of PelA (Colvin et al. 2013). The *N*-deacetylation of GalNAc results in the polymer acquiring a positive charge. Subsequently, the polymer is exported to the extracellular space through the β-barrel outer membrane porin PelB (Marmont et al. 2017). The mechanism by which Pel remains associated with the cell is unclear, although the hydrolase domain of PelA is important for generating a non-cell-associated form of Pel (Razvi et al. 2023).

Knowledge of Pel's structure opened doors to determine its adhesion mechanism and its role in biofilm formation and disease. A screening of lectins targeting specific sugar moieties identified the wisteria floribunda lectin (WFL), which binds the *N*-acetylgalactosamine sugars in Pel. A method for the direct visualization of Pel in biofilms was developed by combining Pel-specific fluorescently labeled WFL-lectin staining with confocal microscopy. Pel localized to two distinct regions in the biofilm: the biofilm 'stalk' near the base of the biofilm and the periphery of the aggregate (Jennings et al. 2015). Periphery staining of biofilm aggregates was strain dependent such that in biofilms without Psl, Pel localized to the periphery (Jennings et al. 2015). The results suggest that Psl is the primary matrix component at the biofilm periphery, but that Pel can compensate for the lack of Psl, providing a layer of redundancy in biofilm formation.

Localization of Pel to the biofilm stalk occurred in all tested *P. aeruginosa* strains, even in strains constitutively expressing Pel, suggesting that transcriptional control alone could not explain the observation. Another essential biofilm matrix component that localizes to this same region in the biofilm stalk is eDNA, which is released by the lysis of a subpopulation of cells at the center of mature biofilm aggregates (Allesen-Holm et al. 2006). Co-localization of Pel and eDNA in *P. aeruginosa* biofilm aggregates suggested an interaction between the polymers. To test this, Pel from culture supernatant was combined with salmon sperm DNA in an in vitro aggregation assay. The binding of Pel to DNA through the crosslinking of polymers was visualized by the formation of aggregates. These in vitro aggregation assays demonstrated that Pel binds salmon sperm DNA at a pH where Pel is positively charged (pH < 6.9). Taken together, the results indicate that Pel binds eDNA in biofilms via an ionic binding mechanism (Jennings et al. 2015). Charge-based interactions are an important mechanism of adhesion for Pel allowing it to bind eDNA and other disease-relevant anionic polymers including mucin and hyaluronan (Jennings et al. 2015).

While much of our understanding of biofilms comes from in vitro experiments, questions remain about the relevance of these findings to infections in vivo. CF is a genetic disorder that predisposes individuals to *P. aeruginosa* lung infections

(Davies 2002). Individuals with CF routinely expectorate sputum, which contains mucin and bacteria from the CF airways (Nicholas and Djukanović 2009). Sputum can be utilized as a window into CF microbiology to determine if biofilm polysaccharides are produced in the CF airways. To answer this question, a method for the direct visualization of bacterial exopolysaccharides in CF sputum was developed using immunohistochemistry with Pel- and Psl-specific antibodies. Immunohistochemistry results reveal that Pel and Psl are associated with *P. aeruginosa* aggregates in CF sputum, compared to controls without *P. aeruginosa* and isotype controls (Fig. 1.8) (Jennings et al. 2021). All sputum samples containing *P. aeruginosa* produced Pel, Psl or both. Pel staining was more prevalent than Psl staining and detected in five of five sputum samples with *P. aeruginosa*. Psl was detected in three of five *P. aeruginosa*-containing sputum samples. The presence or absence of bacterial polysaccharides in sputum was consistent with DNA evidence from PCR and the proposed bacterial aggregation mechanism (see the next paragraph). In vivo aggregates shared features with in vitro biofilms, such as a matrix that extends beyond the cell cluster. Generally, sputum aggregates were smaller than aggregates typically found in flow-cell biofilms, possibly due to nutrient or growth limitations. This direct evidence from CF sputum supports the notion that biofilm matrix exopolysaccharides are indeed produced at sites of human infection.

The formation of aggregates by *P. aeruginosa* in the CF airways is well-established (Bjarnsholt et al. 2009; Singh et al. 2000), but how the aggregates form in vivo is controversial and unclear. The debate revolves around whether the environment or bacterial polysaccharides drive bacterial aggregation. Understanding this mechanism is crucial, as physically disrupting aggregates restores their sensitivity to antibiotics and immune cells (Alhede et al. 2011; Kharazmi 1991). One

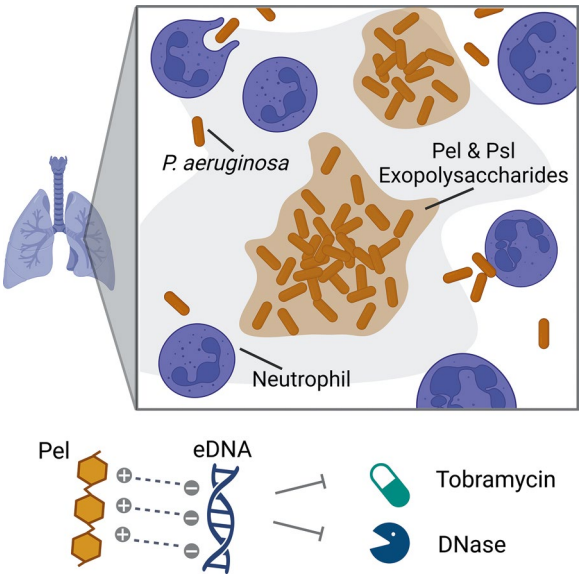

Fig. 1.8 Top: Pel and Psl exopolysaccharides are produced by *P. aeruginosa* aggregates in CF sputum. Bottom: Pel interactions with eDNA increase tolerance to antibiotic and mucolytic treatments

proposed mechanism is depletion aggregation, where the polymer-rich environment causes bacterial aggregation, providing more space for the polymers and increasing the entropy of the system (Secor et al. 2018; Dorken et al. 2012). Depletion aggregates are characterized by a lateral alignment of the cells (the long side of rod-shaped bacteria lines up). Depletion interactions are relatively weak, and the aggregates dissolve upon dilution of the polymers. Another mechanism of bacterial aggregation is polymer bridging, where polymers such as polysaccharides physically connect bacterial cells, forming a "bridge" between them (Strand et al. 2003). Polymer-bridging aggregates show a random or non-lateral alignment of cells, with denser regions of bacteria toward the center. Forces within polymer-bridging aggregates are strong and not easily disrupted by polymer dilution.

To determine the aggregation mechanism in CF sputum, the cellular orientation and packing of bacterial aggregates were assessed. Aggregate morphology in sputum was characterized by the non-lateral alignment of cells and increased bacterial density toward the center of the aggregate, consistent with a polysaccharide-dependent aggregation mechanism, such as by Pel or Psl (Fig. 1.9) (Jennings et al. 2021). Further support for this mechanism comes from the lack of dissolution of bacterial aggregates in sputum upon dilution. While these findings suggest a predominant polysaccharide-dependent mechanism, the data do not rule out the possibility of multiple aggregation mechanisms in sputum. These findings have important implications for antibacterial therapeutics, suggesting that targeting biofilm polysaccharides may be effective at disrupting aggregates in CF.

It was hypothesized that the ability of Pel to bind eDNA in biofilm aggregates may interfere with CF therapeutics. Two common treatments for CF are the antibiotic tobramycin and aerosolized recombinant human DNase used to digest eDNA, reduce sputum viscosity, and improve airway clearance and lung function (Shak et al. 1990). Conditions in CF airways favor Pel binding to DNA due to the abundance of eDNA from lyzed neutrophils (Lethem et al. 1990; Kirchner et al. 1996)

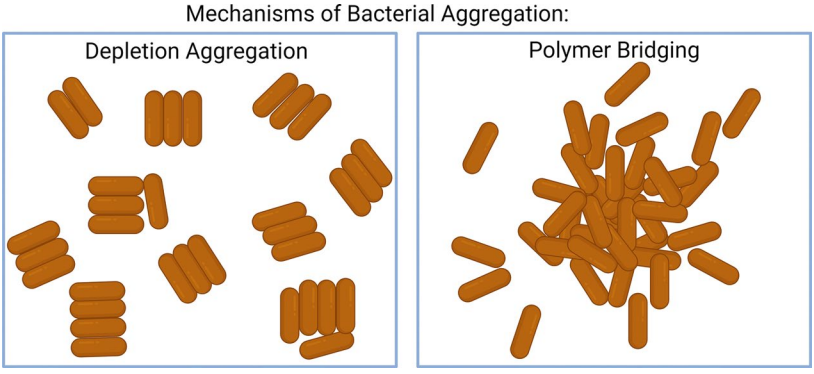

Fig. 1.9 Mechanism of bacterial aggregation in CF sputum is consistent with a polymer-bridging mechanism, such as by Pel and Psl polysaccharides. Cellular orientation in CF aggregates is not laterally aligned. Cells tend to be denser toward the center of the aggregate. Aggregates do not dissolve upon dilution

and the acidic pH, where Pel would be positively charged (Garland et al. 2013). To investigate the influence of Pel–eDNA interactions on antibiotic susceptibility, a static colony biofilm assay was used. Pel-rich colony biofilms supplemented with salmon sperm DNA exhibited reduced susceptibility to tobramycin, with protection observed only with the positively charged antibiotic tobramycin and not the neutral antibiotic ciprofloxacin (Jennings et al. 2021). The results indicate that exogenous DNA enhances tolerance to tobramycin in biofilms with Pel (Jennings et al. 2021). This is consistent with the observation that eDNA increases tolerance to aminoglycosides by multiple mechanisms (Chiang et al. 2013; Wilton et al. 2016).

To explore whether Pel interactions with DNA provide protection against enzyme digestion, Pel aggregates with salmon sperm DNA were subjected to digestion with DNase I, and DNA was visualized with ethidium bromide staining in an agarose gel. While soluble DNA was readily digested by DNase, extended digestion times (up to 48 h) were insufficient to degrade DNA bound to Pel, indicating that Pel protects eDNA from nuclease digestion (Jennings et al. 2021). Collectively, this work sheds light on the biological function of bacterial exopolysaccharides in vivo. Moreover, it provides some of the first direct evidence that Pel and Psl are produced in the CF airways where they likely diminish the efficacy of antibiotic and mucolytic treatments.

This research suggests that targeting Pel- and Psl-mediated aggregation may disrupt *P. aeruginosa* biofilms in vivo. Enzymes capable of degrading biofilm matrix exopolysaccharides Pel and Psl have been proposed as antibiofilm therapeutics. Specifically, the glycoside hydrolases $PelA_h$ and $PslG_h$, encoded in the *pel* and *psl* biosynthetic operons, respectively, selectively degrade Pel and Psl polysaccharides. Treatment with these hydrolases led to a significant reduction in biofilm biomass from clinical and environmental isolates of *P. aeruginosa*, ranging from 58 to 94% (Baker et al. 2016). Additionally, these hydrolase enzymes demonstrated a synergistic effect when combined with antibiotics, enhancing neutrophil-mediated killing. The application of glycoside hydrolases in treating biofilms emerges as a potential alternative for addressing drug-resistant biofilms.

1.5.4 PNAG: Ubiquitous Biofilm Constituent and Prime Immunization Target

Poly-*N*-acetylglucosamine (PNAG), previously known as polysaccharide intercellular adhesin (PIA), is an exopolysaccharide that plays a vital role in bacterial adhesion to surfaces and the formation of biofilm structures. PNAG consists of a homopolymer of β-1,6-*N*-acetylglucosamine, with a degree of deacetylation ranging from 10 to 20% (Mack et al. 1996). Strain-specific variations may occur in the number of *O*- and *N*-linked acetates and *O*-linked succinates (Atkin et al. 2014; Skurnik et al. 2016). PNAG exists in both non-cell-associated and cell-associated

forms, with molecular masses ranging from 21 to 460 kDa and up (Maira-Litrán et al. 2002).

PNAG was first discovered in *Staphylococcus epidermidis* in a screen of transposon mutants that were deficient in biofilm formation (Mack et al. 1994). *S. epidermidis* is an opportunistic pathogen and a common cause of nosocomial and indwelling medical device-related infections (Vuong and Otto 2002). PNAG was subsequently identified in other pathogenic bacteria including *S. aureus* (Cramton et al. 1999), *E. coli* (Wang et al. 2004), *Yersinia pestis* (Yoong et al. 2012), *Bacillus subtilis* (Roux et al. 2015), *Acinetobacter baumannii* (Choi et al. 2009), and *Bordetella pertussis* (Conover et al. 2010).

Phylogenetic analysis of PNAG loci reveals homologues in numerous bacterial species, with some, like *E. coli* and *Y. pestis*, possessing complete PNAG loci, while others, including *Salmonella*, *Shigella*, *P. aeruginosa*, and *V. cholerae*, lack an evident PNAG locus (Wang et al. 2004). Within *S. aureus*, most clinical isolates encode PNAG, emphasizing its widespread prevalence and clinical relevance (Fowler Jr. et al. 2001). Immunochemical detection using monoclonal antibodies specific to PNAG indicates its broad conservation among various bacterial, fungal, and protozoal pathogens, some of which lack an obviously identifiable PNAG locus (Cywes-Bentley et al. 2013). These results suggest that PNAG is broadly conserved among diverse bacteria, leading to its consideration as a potential antibacterial vaccine candidate (Skurnik et al. 2016).

The synthesis of PNAG is governed by both environmental conditions and phase variation. While many strains have the capability to produce PNAG, its expression is contingent on various growth conditions, including anaerobic growth, high temperature, osmolarity, exposure to sub-MIC antibiotics, or other stresses (Cue et al. 2012; Limoli et al. 2015; Rachid et al. 2000). Independent of environmental conditions, the transcription of the *icaADBC* operon is subject to phase variation, a process involving random and reversible switches in gene expression, often mediated by replication slippage at tandem repeats within gene coding regions (Brooks and Jefferson 2014). Specifically, slipped-strand mispairing at a tetranucleotide tandem repeat within *icaC* frequently and reversibly inactivates IcaC, leading to the loss of PNAG production. The authors suggest that the ability to randomly toggle PNAG production on and off may confer a selective advantage to the bacteria. This is because high-level PNAG production is energetically costly and may only be advantageous under specific conditions. The dynamic regulation of PNAG synthesis, influenced by traditional sense-and-response mechanisms triggered by environmental cues and random alterations in gene expression resulting from phase variation, enables bacteria to vary their PNAG production, optimizing resource allocation for maximum fitness.

PNAG is predicted to be synthesized by a synthase-dependent mechanism, similar to Pel (Low and Howell 2018). In *S. epidermidis* and *S. aureus*, PNAG synthesis is governed by the *icaADBC* operon and inhibited by *icaR* (Heilmann et al. 1996; Cramton et al. 1999). The glycosyltransferase IcaA is responsible for polymerizing PNAG, utilizing UDP-*N*-acetylglucosamine as a substrate (Atkin et al. 2014). The inner membrane protein IcaD is thought to enhance the glycosyltransferase's

efficiency. While it is suggested that IcaC may be involved in translocation or potentially act as an *O*-succinyltransferase to modify PNAG's structure, its precise function remains unknown (Atkin et al. 2014). Subsequently, the polymer undergoes transportation through the periplasmic space, where IcaB catalyzes de-*N*-acetylation.

In Gram-negative *E. coli*, PNAG synthesis occurs through the homologous *pgaABCD* locus (Wang et al. 2004). The PgaC glycosyltransferase is predicted to catalyze the polymerization of 1-6-*N*-acetylglucosamine using UDP-*N*-acetylglucosamine as a precursor (Whitney and Howell 2013). The resulting polymer is fully acetylated before undergoing transport across the inner membrane. The functions of inner-membrane proteins PgaC and PgaD remain unknown. Subsequently, the polymer undergoes de-*N*-acetylation in the periplasm by PgaB before being exported to the extracellular space through PgaA, a predicted outer-membrane protein (Whitney and Howell 2013). This partial deacetylation of PNAG holds significance for its interactions with the negatively charged bacterial surface, as well as its role in biofilm formation and immune evasion (Ostapska et al. 2018).

PNAG plays a crucial role in mediating the aggregation of bacteria into biofilms. Its significance is particularly pronounced in the later stages of biofilm formation, such as microcolony development and maturation rather than initial attachment. This is evident from observations that *ica* deletion mutants can successfully aggregate, but not attach to surfaces (Cramton et al. 1999), and that *S. aureus* and *S. epidermidis* primarily utilize cell surface proteins for adherence to the host extracellular matrix (Patti et al. 1994). The importance of PNAG in biofilm formation varies among strains, much like the strain-dependent reliance on *P. aeruginosa* exopolysaccharides. While some strains heavily depend on PNAG for biofilm formation, others employ protein- and/or DNA-rich matrices for this purpose (Limoli et al. 2015). Notably, *S. epidermidis* demonstrates a unique capability to switch between a polysaccharide-dependent matrix and one rich in proteins (Hennig et al. 2007). Research by Vuong et al. underscores the significance of PNAG's cationic charge in influencing many biofilm aggregation phenotypes (Vuong et al. 2004a). Collectively, this highlights the diverse and dynamic nature of biofilm formation, where the role of PNAG is intricately linked to biofilm aggregation and can vary across bacterial strains.

PNAG serves as a critical virulence factor for numerous bacterial pathogens. Mutants deficient in PNAG display attenuated virulence in animal models of infection. In *S. aureus*, PNAG is a virulence factor in multiple murine models of systemic infection including bacteremia, renal abscess formation, and lethality following high-dose intraperitoneal infection (Kropec et al. 2005). Additionally, in *S. epidermidis*, the cationic charge of PNAG proves essential for the colonization to host epithelial cells, highlighting a potential role in the initiation of infection (Vuong et al. 2004a). In uropathogenic *E. coli*, transposon mutants in PNAG biosynthesis genes result in reduced pathogen fitness in murine models of urinary tract infections and systemic infections (Subashchandrabose et al. 2013). Furthermore, PNAG is implicated in the pathogenesis of device-related infections; as a PNAG mutant is less likely to cause infection of central venous catheters in a rat model of infection

(Rupp et al. 2001). Diverse models of infection highlight the importance of PNAG in pathogenesis.

PNAG promotes persistent infections by increasing resistance to the immune system and antimicrobials. PNAG on the surface of bacteria can conceal detection of pathogen-associated molecular patterns (PAMPs) from recognition by the innate immune system (Ostapska et al. 2018). PNAG can also prevent elimination of pathogens by immune cells. In *S. epidermidis*, the removal of PNAG from the bacterial surface makes the pathogen more prone to phagocytosis by human neutrophils (Vuong et al. 2004a). The PNAG-mediated resistance to opsonic killing is thought to be mediated by preventing complement opsonins from binding to the bacterial surface or preventing complement receptors on phagocytes from binding opsonins (Skurnik et al. 2016). Biofilms with PNAG as a key component often exhibit increased resistance to antimicrobials. PNAG can provide protection from antimicrobials including the cationic detergent cetylpyridinium chloride in *Aggregatibacter actinomycetemcomitans* (Izano et al. 2008), vancomycin antibiotics, and the antimicrobial peptide dermcidin in *S. epidermidis* (Farber et al. 1990; Vuong et al. 2004b).

Research on PNAG's role in biofilm formation and its impact on infection pathogenesis has facilitated the design and testing of antibacterial therapies targeting this polysaccharide. Multiple studies have indicated that PNAG is upregulated during infection, including its presence in CF sputum containing *S. aureus* (Mckenney et al. 1999) and middle ear effusions from *H. influenzae*- or *S. pneumoniae*-infected otitis media samples (Cywes-Bentley et al. 2013), making it a promising vaccine candidate. Natural antibodies to PNAG are commonly detected in humans and animals, but they fail to protect against PNAG-producing bacteria (Skurnik et al. 2012).

Vaccination with PNAG, particularly with an elevated level of deacetylation, has shown promising results, eliciting high levels of immunity across diverse bacteria producing PNAG in early clinical trials (Skurnik et al. 2016; Lu et al. 2023). Partial deacetylation of PNAG is needed for retention of the polymer on the bacterial cell surface. The surface retention of PNAG in *S. aureus* is key to explaining the protective activity of the antibody to highly deacetylated PNAG (Cerca et al. 2007). This suggests that non-cell-associated PNAG may act as a decoy, binding opsonic antibodies and preventing them from mediating killing at the pathogen cell surface. Immunization with deacetylated PNAG shows promise as an antibacterial therapeutic. Future work is required to determine if the strategy would be effective on the diverse pathogens that produce PNAG.

1.6 Summary and Future Perspectives

In summary, the examination of exopolysaccharides in the bacterial biofilm matrix, specifically alginate, Pel, Psl, cellulose, and PNAG, highlights their multifaceted roles from initial bacterial adhesion to the establishment of mature biofilms. The tightly regulated synthesis of exopolysaccharides showcases the biofilms' adaptability to varied environmental conditions. This adaptability enables bacteria to

modify their exopolysaccharide production based on specific cues, optimizing resource allocation for maximum fitness. A common subset of exopolysaccharides produced by diverse bacteria suggests these particular polymers harbor important functions in biofilms and disease. However, the variation in exopolysaccharides among bacterial strains underscores the complexity of biofilm formation mechanisms and the need for a nuanced understanding of strain-specific variations.

Exopolysaccharides significantly contribute to infection pathogenesis in biofilm-forming bacteria. Many mutants lacking exopolysaccharides exhibit attenuated virulence, underscoring their role in infections. The ability of exopolysaccharides to evade immune detection and resist opsonic killing further emphasizes their importance in promoting persistent infections. The resistance of exopolysaccharide-containing biofilms to conventional antimicrobials complicates treatment options. Because of this, developing targeted therapies to disrupt exopolysaccharide synthesis or enhance immune recognition is a promising approach for combating biofilm-associated infections. Encouraging results from early clinical trials involving vaccination with PNAG, particularly with elevated deacetylation levels, underscore the potential of exopolysaccharides as vaccine candidates. Other approaches utilizing hydrolase enzymes to degrade exopolysaccharides and disrupt biofilms offer attractive treatment alternatives for biofilm infections. Future research should focus on innovative antibacterial therapeutic strategies, assessing their efficacy across diverse bacterial species.

Looking ahead, a deeper mechanistic understanding of exopolysaccharide-mediated biofilm dynamics is crucial. Research should explore the intricate interplay between different exopolysaccharides, matrix proteins, and bacterial surface structures. Understanding how these elements interact within the biofilm matrix will enhance our comprehension of biofilm dynamics. In addition, unraveling host–pathogen interactions at the interface of biofilms and the immune system is imperative for targeted therapeutic interventions. Beyond the clinical realm, exploring how exopolysaccharides contribute to biofilm formation in various ecological niches can provide insights into broader implications. Bridging these knowledge gaps surrounding exopolysaccharides will advance our understanding of bacterial biofilm biology and inspire innovative therapeutic strategies for biofilm-related infections.

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