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Bacterial Extracellular Polysaccharides in Biofilm Formation and Function

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Abstract

Microbes produce a biofilm matrix consisting of proteins, extracellular DNA, and polysaccharides that is integral in the formation of bacterial communities. Historical studies of polysaccharides revealed that their overproduction often alters the colony morphology and can be diagnostic in identifying certain species. The polysaccharide component of the matrix can provide many diverse benefits to the cells in the biofilm, including adhesion, protection, and structure. Aggregative polysaccharides act as molecular glue, allowing the bacterial cells to adhere to each other as well as surfaces. Adhesion facilitates the colonization of both biotic and abiotic surfaces by allowing the bacteria to resist physical stresses imposed by fluid movement that could separate the cells from a nutrient source. Polysaccharides can also provide protection from a wide range of stresses, such as desiccation, immune effectors, and predators such as phagocytic cells and amoebae. Finally, polysaccharides can provide structure to biofilms, allowing stratification of the bacterial community and establishing gradients of nutrients and waste products. This can be advantageous for the bacteria by establishing a heterogeneous population that is prepared to endure stresses created by the rapidly changing environments that many bacteria encounter. The diverse range of polysaccharide structures, properties, and roles highlight the importance of this matrix constituent to the successful adaptation of bacteria to nearly every niche. Here, we present an overview of the current knowledge regarding the diversity and benefits that polysaccharide production provides to bacterial communities within biofilms.

INTRODUCTION

The ability to construct and maintain a structured multicellular bacterial community depends critically on the production of extracellular matrix components (1, 2). While the biofilm matrix may be composed of various molecules, the focus of this chapter is on the extracellular polysaccharides (PSs) important for biofilm formation.

The PSs synthesized by microbial cells vary greatly in their composition and hence in their chemical and physical properties. Many are polyanionic, but others are neutral or

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polycationic (see Table 1 and Fig. 1) (2). In most natural and experimental environments, PSs are found in ordered compositions, with long, thin molecular chains, ranging in mass from 0.5 to 2.0 × 10^6 Da. PSs can be elaborated in a multitude of ways, influenced by the environment and association with other molecules such as lectins, proteins, lipids, and bacterial and host extracellular DNA (eDNA). Moreover, many biofilms are composed of multiple bacterial or even fungal species, whereby a range of PSs may interact to generate further permutations of unique architectures (3).

The diversity in PS structure also provides a range of functional roles for PSs in microbial biofilms. For many bacteria, structural and physical consequences of PS expression confer unique colony morphology phenotypes (Fig. 2). The PS in the biofilm matrix dictates a framework for the biofilm landscape. Inhabitants of the biofilm need to be protected from the environment (host cells, antimicrobials, desiccation, temperature, competing microbes, etc.) while maintaining access to nutrients and the ability to respond to changes in the environment. Bacteria generate multiple PSs to cope with these needs in a variety of different ways. PSs can help bacteria adhere to a multitude of different surfaces and host and bacterial cells, provide protection from the onslaught of antimicrobials in the environment, provide reservoirs for nutrient acquisition, and aid in the creation of distinct architectures, which further potentiate an environment suitable for microbes to persist. In this chapter, we will discuss PSs that are known to be important for microbial biofilm formation. For strictly organizational purposes, PSs are divided here into three functional categories to highlight their importance and diversity in biofilm biology. While these PSs are subjectively categorized into aggregative, protective, and architectural, these divisions are by no means exclusive. Several PSs have roles in each of these categories (see Table 1), which will also be discussed below.

AGGREGATIVE POLYSACCHARIDES

The formation of biofilms occurs in multiple stages: initial attachment, microcolony and macrocolony formation, and detachment or disassembly (4–6). Aggregative PSs play essential roles in each of these steps: aiding in adhesion to surfaces, formation of complex structures by promoting microbial interactions, and relief of these interactions promoting dissolution of the biofilm. Bacteria can elaborate multiple PSs, which are important in different strains and varying environmental conditions, including surface substrate, nutrition, and flow rate (7). The redundancy of aggregative PSs produced by many bacteria highlights the essentiality of bacteria remaining associated with the biofilm community. Moreover, the ability to modify PS production provides compensatory mechanisms to adapt to changing environments. The PSs described in this section highlight the importance of aggregation in the biofilm community lifestyle and demonstrate the range of functions of these PSs.

Polysaccharide Intercellular Adhesion

Significance, structure, and regulation—The PS intercellular adhesion (PIA) is the primary PS involved in biofilm formation of *Staphylococcus aureus* and *Staphylococcus epidermidis*, which contribute significantly to endocarditis, osteomyelitis, and infections associated with indwelling medical devices (4, 8). PIA was originally identified in *S. epidermidis* and named as such due its role in mediating cell-cell interactions during biofilm

formation (9–11). Similar PSs were subsequently identified in *S. aureus* and referred to as polysaccharide/ adhesion (PS/A or PSA) (12), poly-N-acetylglucosamine (13) or *S. aureus* exopolysaccharide (14). It has since been verified that despite possible variations in the degree of N-acetylation, O-succinylation, and molecular weight, each PS represents the same homopolymer composed of β-1-6 linked 2-amino-2-deoxy-D-glucopyranosyl residues (Fig. 1) (11, 13, 15). PIA is synthesized by enzymes encoded by the *icaADBC* locus, which is negatively regulated by *icaR* located upstream of *icaA* (16–18). Importantly, *icaADBC* orthologous genes have also been identified in other human pathogens such as *Escherichia coli* (19), *Yersinia pestis* (20), *Aggregatibacter actinomycetemcomitans, Actinobacillus pleuropneumoniae* (21), *Bordetella* spp. (22), *Klebsiella pneumoniae, Enterobacter cloacae, Stenotrophomonas maltophilia*, the *Burkholderia cepacia* complex (BCC) (23), and *Acinetobacter baumannii* (24). The *ica* operon is tightly regulated *in vitro* and is induced by a number of environmental conditions. Although there is strain-to-strain variation, PIA can be induced in the presence of high NaCl, glucose, temperature and ethanol, anaerobiosis, and subinhibitory concentrations of some antibiotics (25–27). In *S. epidermidis* the quorum sensing LuxS system negatively regulates PIA expression, where a *luxS* mutant demonstrates increased biofilm formation and enhanced virulence in a rat model of biofilm-associated infection (28). σ^B and RsbU also positively regulate PIA. σ^B activates the *ica* operon by repressing transcription of icaR though an unknown intermediate (29). In *S. aureus* the Spx protein, which directly interacts with RNA polymerase to regulate transcription, negatively regulates the *ica* operon and biofilm formation (30, 31), while the global regulator SarA positively regulates PIA production and biofilms (32, 33). The importance of PIA during staphylococci infections has been extensively studied, and several roles have been attributed to PIA, such as promoting cellular aggregation and biofilm formation, increased virulence, and protection from host innate immune responses (34–36; reviewed in references 25, 37–40).

Role in biofilm biology—In *S. aureus* and *S.epidermidis*, initial attachment is mediated primarily by cell surface proteins that bind to mammalian extracellular matrix/plasma proteins (41). The micro- and macro- colony formation stage requires intercellular bacterial aggregation, and in staphylococci, this is mediated primarily by PIA. This has been illustrated by mutants in the *ica* operon retaining the ability to adhere to surfaces while being incapable of forming multilayered biofilms (16). In the detachment stage of the biofilm lifestyle, matrix components are often degraded; however, staphylococci do not seem to possess PIA hydrolytic enzymes (37). Instead, PIA is dispersed by elevating expression of detergent-like peptides, which disrupt noncovalent interactions between PIA and the bacterial cell surface (38). The importance of PIA for staphylococci biofilm formation has been demonstrated *in vivo* in several animal models where the *ica* operon is upregulated and required for biofilm formation during infection (42–46). However, staphylococcal strains demonstrate a wide range of biofilm phenotypes, and many strains maintain the ability to form biofilms in the absence of PIA (32, 47, 48), where several PIA-independent protein factors and/or eDNA seem to be more important (8, 49, 50). At least some strains are also able to switch between PIA-dependent and -independent phenotypes, which may aid in adaptation to changing environments (51). Collectively, these data demonstrate that biofilm

formation and PIA production are integral to the staphylococcal lifestyle and its ability to cause a range of diseases.

Pseudomonas aeruginosa Pel

Significance, structure, and regulation—Pel is an aggregative PS produced by *P. aeruginosa*. It derives its name from the thick pellicle observed in strains overexpressing the *pel* operon. This operon encodes seven enzymes with homology to other PS synthesis proteins; however, several genes predicted to be essential for Pel production are missing from this operon (52–54). This indicates that there may be reliance on several other PS synthesis enzymes encoded elsewhere on the chromosome. The structure and composition of Pel remain unknown, but studies are underway to identify the sugars and linkages present in this PS. The common strain PA14 relies exclusively on Pel production for aggregation, because it is missing the *pslABCD* genes (see below) (52).

Pel production is increased by elevated amounts of the intracellular second messenger cyclic diguanylate (c-di-GMP) (55). Recent studies have described a role for the flagellum regulator FleQ in the regulation of the *pel* operon, where the cellular pool of c-di-GMP is sensed by FleQ, which causes FleQ to change from a repressor of the *pel* promoter to an activator (56, 57). In addition, c-di-GMP functions post-translationally in Pel synthesis by modulating activity of PelD (55).

Role in biofilm biology—The contribution of Pel to biofilm structure is most evident when observing biofilms that form at the air-liquid interface, called pellicles. The production of Pel is strongly correlated with increased pellicle formation, adherence to culture tubes, and formation of aggregates in broth culture (52, 58, 59). The biofilm properties of Pel and Psl (below) are closely linked, because there is evidence of functional overlap in these two PSs (59). It appears that the structural requirements of these two PSs for biofilm formation are strain specific, because there are examples of strains which are incapable of producing one or the other yet still can efficiently form biofilms (59).

Pseudomonas aeruginosa Psl

Significance, structure, and regulation—The polysaccharide synthesis locus produces another important *P. aeruginosa* PS, Psl (60). The *psl* operon consists of 15 genes (*pslA-O*), which are cotranscribed (61). Mutagenesis studies revealed that 11 of these genes (*pslACDEFGHIJKL) are* essential for Psl production and surface attachment (62). There are two forms of Psl, a high molecular weight cell-associated form and a low molecular weight form that appears to be released from cells (62). Released Psl consists of D-mannose, D-glucose, and L-rhamnose in a 3:1:1 ratio, respectively (Fig. 1) (62, 63). The structure of cell-associated Psl is unknown but is believed to be a polymer of mannose, glucose, and rhamnose and possibly galactose (63). Psl is produced primarily in nonmucoid strains, because expression of the *psl* operon is repressed in mucoid strains (64).

The effect of Psl is most easily studied in rugose small colony variant strains such as the Δ*wspF* mutant, where Psl is overproduced (65). The overproduction of Psl leads to a rough, wrinkled colony morphology and hyper-biofilm phenotype, which is consistent with

overexpression of adhesive PSs in other bacteria (66, 67). As with Pel, high expression of Psl in this strain is due to elevated levels of the second messenger cyclic di-GMP (65, 68, 69). There are several pathways that lead to regulation of Psl production by altering the c-di-GMP level of the cell. The first of these regulatory pathways to be described was the regulation of the *psl* operon by the master flagellum regulator, FleQ (56, 57). In this instance, c-di-GMP relieves the repressive effect of FleQ at the *psl* operon promoter, leading to expression of the *psl* operon (56, 65).

Another important observation is that patients who survive systemic infections with *P. aeruginosa* often produce neutralizing antibodies against Psl (70). Passive immunization with these antibodies is protective against *P. aeruginosa* infection in several murine models (70). Psl also increases adherence to epithelial cell layers and evasion of phagocytosis by neutrophils, emphasizing that Psl plays important roles during the initiation and persistence of chronic infections (71, 72).

Role in biofilm biology—PAO1 Δ*psl* mutants form thin, diffuse biofilms lacking structure compared to the wild type PAO1, while the *psl* overexpressing strains form thicker biofilms with significantly more biomass and microcolony height (73). While this suggests that Psl is essential for biofilm formation in *P. aeruginosa* strain PAO1, it is important to note that this requirement of Psl for biofilm formation varies from strain to strain. For example, the common strain PA14 does not synthesize Psl yet retains the ability to form biofilms (74). This discovery led to an investigation of the roles of the adhesive PSs, Psl, and Pel in various *P. aeruginosa* isolates. It was concluded that there are strain-specific requirements for PS production in biofilms. In some strains, Psl is exclusively required for biofilm formation, while in other strains, Psl and Pel are functionally redundant (59). Importantly, one of these two adhesive PSs is required in all strains tested to form a mature biofilm *in vitro* (59). Recent work indicates that Psl can also function as a signaling molecule that stimulates biofilm formation (75). Psl is also protective against many classes of antibiotics, serving yet another role in the biofilm matrix (76). Psl-producing cells were more resistant to a wide range of antibiotics than cells unable to produce Psl. This protective effect was transferable to cells unable to produce Psl when grown in coculture, including other species of bacteria such as *E. coli* and *S. aureus* (76). Collectively, these observations indicate that Psl is important for initial attachment, resistance, and biofilm structure.

PROTECTIVE POLYSACCHARIDES

Microbial protection from the onslaught of host and environmental factors is one of the most quintessential and frequently described attributes of the biofilm mode of growth. In fact, a current criteria used to define infectious biofilms is increased recalcitrance to antimicrobials and the host immune response (77, 78). While several factors including metabolic heterogeneity, altered chemical microenvironments, and persister cell populations contribute to biofilm antimicrobial resistance, an important role for PSs is clearly evident (79–81). It had been initially proposed that PSs in the matrix pose a diffusion barrier, which protects biofilm-grown cells from antibiotic penetration (82). However, studies have demonstrated that several antibiotics can readily penetrate the biofilm (83, 84). While diffusion may not be prevented, it may be delayed enough to induce expression of genes that mediate tolerance

(85) or be neutralized by enzymes within the matrix before reaching the bacterial cells (86, 87).

Another prevalent and important role for PSs is protection from opsonic and nonopsonic phagocytosis. Several studies demonstrate that antibodies or inflammatory cells cannot efficiently penetrate biofilms embedded in a PS matrix (8, 49, 88–91). Protection from the immune response results in poor detection, promoting maintenance of stable chronic infections and decreased virulence. Importantly, PS-producing bacteria may confer resistance to nonproducing bacteria within the biofilm (76). PSs are also important for providing a hydrated biofilm environment, which can provide protection from desiccation and promote biofilm fluidity (2, 92). The PSs described in this section provide evidence for a diversity of protective functions toward biofilm bacteria.

Alginate

Significance, structure, and regulation—The first *P. aeruginosa* PS described was alginate due to its prevalence in cystic fibrosis (CF) pulmonary infections, whereby up to 90% of *P. aeruginosa* clinical isolates overproduce alginate (referred to as mucoid) (60, 93, 94). CF patients are initially colonized by nonmucoid *P. aeruginosa* (Psl and/or Pel producing) but convert to the mucoid phenotype during the course of infection. Mucoid conversion confers a selective advantage to *P. aeruginosa* in the CF lung (discussed further below) (95). Mucoid conversion is directly correlated with an increase in morbidity and mortality of CF patients and remains a significant clinical challenge for eradicating *P. aeruginosa* infections (96). While the majority of alginate research has focused on *P. aeruginosa*, alginate production has also been reported in other *Pseudomonads* and *Azotobacter vinelandii* (54, 97). Alginate is a random linear polymer of variably acetylated 1,4-linked β-D-mannuronic acid and its C5 epimer α-L-guluronic acid, originally identified in brown seaweeds (Fig. 1) (98, 99). This acetylated polymer structure yields a highly hygroscopic PS promoting biofilm fluidity and resistance to desiccation (2, 92).

Alginate synthesis is tightly regulated due to the high metabolic cost of production (alginate regulation has been extensively reviewed in references 95, 100–102). In brief, expression of the alginate biosynthetic operon (*algD-A*) is dependent on the alternative sigma factor AlgT (also called AlgU$A\sigma^{E}$/σ^{22}/RpoE). The activity of AlgT is antagonized by the anti–sigma factor MucA, which sequesters AlgT to the cell membrane, preventing interaction with AlgT-dependent promoters (100). Alginate is constitutively overproduced (as seen in mucoid CF isolates) upon acquisition of mutation(s) in the *mucA* gene, which results in a truncated, inactive protein. The mutant MucA is unable to inactivate AlgT, allowing expression of the AlgT regulon, including the alginate operon (95, 100, 103). Additional regulators of alginate production include AlgR, IHF, AlgB, and AmrZ (*algD* transcriptional regulators) and MucB to E and P, AlgW, KinB, and ClpX proteases (regulation of MucA stability) (100, 101, 104–106).

Role in biofilm biology—Early microscopy evidence demonstrated that mucoid *P. aeruginosa* forms microcolonies embedded in an extracellular matrix in the alveoli of CF patients (88, 107). Alginate was initially thought to be essential for *P. aeruginosa* adherence

and biofilm formation, (108–111). However, this paradigm has been challenged by multiple groups who, by comparing isogenic mucoid and nonmucoid strains, demonstrated that alginate is not required for biofilm formation (112–15). These studies, corroborated by more recent work, demonstrate that the Psl and Pel PSs provide attachment and structural support for nonmucoid and mucoid biofilms (116, 117), while alginate contributes to generation of a unique architecture (112, 114, 115). Alginate acetylation further influences mucoid biofilm architecture by promoting cell-cell adhesion (112, 114, 115, 118).

The biofilm architecture constructed by mucoid *P. aeruginosa*, combined with the intrinsic properties of alginate, provide *P. aeruginosa* significant protection from antimicrobials in the microenvironment. Mucoid *P. aeruginosa* biofilms are more resistant to antibiotic treatment *in vitro* (115, 119, 120), and even the most aggressive antibiotic treatments are unable to eradicate mucoid *P. aeruginosa* infections in CF patients (96). Alginate also provides protection from the innate immune response by inhibiting both opsonic and nonopsonic phagocytosis, further influenced by the levels of alginate acetylation (121–125). Protection is additionally afforded by the ability of alginate to scavenge reactive oxygen intermediates (126, 127) and inhibit killing by cationic antimicrobial peptides (128–130). Although antibodies to alginate are found in the sera of chronically infected CF patients, these antibodies fail to mediate opsonic killing of *P. aeruginosa in vitro* (131). Additionally, the anionic nature of alginate promotes cation chelation (Ca^{2+} preferentially) (132). Calcium chelation by alginate biofilms induces type III secretion, whose effectors may provide further protection from host immune responses (133). Contemporary studies further emphasize the protective nature of *P. aeruginosa* alginate, demonstrating that mucoid biofilms contain more viable cells than nonmucoid biofilms and are more resistant to DNase treatment (116, 117). Moreover, compared to nonmucoid isolates, mucoid *P. aeruginosa* maintains *in vitro* biofilm formation capacity and gene expression profiles during chronic lung infection of CF patients (134). This suggests that protection from the CF microenvironment afforded by alginate results in a more stable population over time and may help to explain how clonal mucoid strains can persist for decades in CF patients.

Capsular Polysaccharide

Significance, structure, and regulation—Bacterial capsular polysaccharides (CPSs) are found on the cell surface of a broad range of species. The CPS is tightly associated with the bacterial cell surface via covalent attachments to either phospholipid or lipid-A molecules. CPSs are highly hydrated molecules composed of repeating monosaccharides joined by glycosidic linkages (135). CPSs are extremely diverse, not only in the monosaccharide constituents, but also in glycosidic linkages, branching, and substitutions with noncarbohydrate residues, leading to a nearly unlimited range of structures (two examples in Fig. 1) (136). For example, over 80 CPSs (K antigens) have been identified in *E. coli*, and 93 CPSs (serotypes) in *Streptococcus pneumoniae* (137, 138). Despite the variety of CPS structures, many of the details of polymer synthesis and regulation are shared among Gram-positive and Gram-negative bacteria. Capsule synthesis requires nucleotide diphosphosugar precursors available in the cytoplasm and concludes with assembly of the polymer at the periplasmic face of the plasma membrane (136). The three primary bio-synthetic pathways are termed Wyz dependent, synthase dependent, and ATP-binding

cassette transporter dependent (139). Many CPS-producing bacteria respond to environmental conditions by varying capsule production between a low CPS-producing phase and a high CPS-producing phase, whereby stable phenotypic variants may also be selected for under specific environmental conditions (138, 140, 141). Moreover, CPS plays pivotal roles in bacterial survival in the environment and infection in the host, providing protection from desiccation, opsonophagocytosis, and complement-mediated and cationic antimicrobial peptide-mediated killing (135).

Role in biofilm biology—The complexity of biofilm formation in varying environments and stages of infection, the ubiquity and diversity of CPSs among bacteria, and the propensity of bacteria to synthesize multiple PSs have created a complex and inconclusive view of the role of CPSs in biofilm biology. In general, CPS production is thought to inhibit bacterial adherence and biofilm formation. CPS-deficient strains in *S. pneumoniae, Neisseria meningitidis, S. aureus*, and *Vibrio vulnificans* demonstrate increased adherence to epithelial cells and surfaces and more robust biofilm formation (142–146). CPS is also often downregulated during biofilm formation and upon contact with epithelial cells (143, 147, 148). It is hypothesized that decreased CPS further enhances the quiescent nature often associated with biofilm formation: potentiating immune evasion, decreased virulence, and persistence (142, 143, 149). This is supported by observations that low-CPS-producing strains are more frequently isolated from chronic infections, and high-CPS strains, from acute infections (143, 146).

However, this simplistic, binary view may not illustrate the complete role of CPSs in biofilm formation. Studies have demonstrated that CPSs may be important in mature biofilms, aiding in the maintenance of biofilm size and dispersal. In *V. vulnificans*, CPS was found to be expressed in later stages of biofilm formation and was hypothesized to be synthesized and secreted after biofilms mature and reach a threshold of cell density (150). In support of this, CPS expression in mature biofilms and maintenance of biofilm size is regulated by quorum sensing molecules (151). Similarly, in *S. pneumoniae*, higher CPS expression has been demonstrated in biofilm towers, compared to cells at the biofilm surface (148). Further evidence supporting a role of CPS in biofilm maturation is seen during formation of intracellular biofilm-like communities within bladder epithelial cells during urinary tract infections by uropathogenic *E. coli* (149). Collectively, these data point toward an overall view for multiple pathogens that CPS plays an important, yet incompletely defined role in biofilm biology. Several lines of evidence support an inhibitory role during initial attachment and biofilm formation; however, the ability to then enhance CPS synthesis in later stages may prove to be critical during infection. This clearly requires further study.

Levan

Significance, structure, and regulation—Levans are high molecular mass, neutral homopolymers composed of β-D-fructans with extensive and irregular branching (Fig. 1) (152). Levan production confers a mucoid phenotype to bacteria expressing this and has been described in several plant pathogens (*Pseudomonas syringae, Erwinia amylovora*, and *Bacillus subtilis*) and *Streptococcus mutans* oral biofilms (153–155). Levan is produced exclusively from extracellular sucrose catalyzed by excreted levansucrase (153, 156, 157).

Levansucrase is activated in the presence of sucrose and is frequently regulated by two-component sensor kinases (LadS in *P. syringae*; SacX/SacY and DegS/DegU in *B. subtilis*; RscC/RscB, GacS/GacR (GrrS/GrrA), and EnvZ/OmpR in *E. amylovora*; and CovS/CovR [VicK/ VicR] in *S. mutans* [152, 158, 159]). In *B. subtilis sacB* and *sacC* are involved in levansucrase production and modification. Levansucrase is secreted from the cell by the SecA pathway (160, 161). In *P. syringae* and *E. amylovora* the levansucrase biosynthetic operon is composed of three genes: *iscA-C* (153, 162). Proposed functions for levan include protection from desiccation and bacteriophages, nutrient storage, and virulence (157, 163, 164).

Role in biofilm biology—The role of levan production in biofilms has predominately been described in *S. mutans* dental biofilms. Upon exposure to sucrose, levan accumulates on dental plaques and is catabolized to acid when the environmental sugar is depleted. Prolonged exposure promotes dental carries (165). While levan is not required for *S. mutans* biofilm formation, supplementation of sucrose to biofilms *in vitro* yields thicker structures, which contain more levan and are more resistant to shear stress (92, 166, 167). Interestingly, levan is a water-soluble PS, and levan produced by *B. subtilis* possesses a particularly low viscosity. At typical concentrations where other PSs display gel behavior, levan remains fluid, suggesting that it may not form an adhesive framework (168). In *P. syringae*, levan is not required for biofilm formation and is not responsible for maintenance of biofilm structure. Instead, levan may function as a storage molecule, utilized during periods of starvation (164). Levan was identified in the voids and blebs of *P. syringae* biofilms, whose formation required supplementation of sucrose in the media (164). This theory of nutrient retention by levan was previously suggested for *S. mutans* in the oral cavity (163). Collectively, these data suggest that levan contributes to the physio-chemical properties of bacterial biofilms but is not required for its formation.

ARCHITECTURAL POLYSACCHARIDES

Some of the first biofilm-related PSs identified were studied due to the role that they play in biofilm structure. These PSs have provided extensive insight into the regulation of biofilm formation and structure, primarily due to the readily observable phenotypes associated with mutants or overproducers of these products. For example, through studying the rugose phenotype associated with PS produced by *Vibrio cholerae* (*Vibrio* polysaccharide [VPS]), important studies in gene regulation, second messenger signaling, and biofilm matrix assembly have been reported (169–171).

Colanic Acid

Significance, structure, and regulation—Colanic acid (CA), or M antigen, is a branched PS composed of glucose, galactose, and glucuronic acid (Fig. 1) (172). The 19-gene *wca* cluster is responsible for producing CA in several species of *Enterobacteriaceae* (172–174). This cluster was formerly called the *cps* cluster, but it was renamed when it was discovered that these genes encode enzymes that produce CA (172). As with most PSs, several of the precursors of CA, such as UDP-D-glucose and UDP-D-galactose are produced by enzymes that are located in other loci, while many of the enzymes dedicated to CA

synthesis are included in the *wca* cluster (172). For many years, the study of CA was focused on its effect on colony morphology, but advances in molecular techniques allowed a genetic approach to the regulation, structure, and role of CA in the biofilm matrix. The *wca* gene cluster is regulated by the well-characterized Rcs phosphorelay system. In this variation of a two-component system, RcsC transfers a phosphate to RcsD, which subsequently transfers it to RcsB. RcsB is a LuxR family helix-turn-helix response regulator, which binds the promoter of the *wca* operon and activates transcription (175–178). The highly unstable accessory protein RcsA can enhance this interaction. Collectively, this signaling cascade leads to upregulation of the *wca* cluster and production of CA, which allows the bacterium to endure environmental stresses such as desiccation (179). CA is primarily produced at lower temperatures, indicating that it is most beneficial to the bacterium in the environmental phase of its life cycle (175).

Role in biofilm biology—CA was one of the first PSs studied with respect to its role in biofilm formation. Early *in vitro* work demonstrated that an *E. coli* mutant incapable of producing CA had two interesting phenotypes. First, the CA mutant demonstrated attachment capabilities similar to the wild type strain. This was interesting to the biofilm field, because previous work on the role of PSs in biofilms indicated that the PS was involved in the initial attachment to surfaces (12, 180). Second, though the CA mutant was able to attach at wild type levels, it formed biofilms with less three-dimensional structure than the biofilms formed by the wild type strain (181). These biofilms were described as "collapsed," with cells densely packed against the substrate. It was concluded that CA was critical for the development of mature, three-dimensional biofilms by *E. coli* (181). This work was integral to the biofilm field, because it was one of the first observations that implicated PSs as structural members of the biofilm matrix.

In *E. coli*, CA is produced at low temperatures and therefore is more important for environmental survival than during infection (182). In contrast, the role of CA produced by the pathogen *Salmonella enterica* serovar *Typbimurium* is dispensable for binding abiotic surfaces (183). Further characterization of the role of CA in *S. enterica* biofilms indicated that the CA mutants were unable to build a three-dimensional biofilm on either HEp-2 cells or chicken intestinal epithelium, suggesting that CA is involved in pathogenesis (183). These divergent roles for CA in two relatively closely related bacteria demonstrate how PSs can enhance the diversity of bacterial colonization niches and biofilm phenotypes.

Vibrio Polysaccharide

Significance, structure, and regulation—*V. cholerae* is a Gram-negative aquatic bacterium that is the causative agent of the diarrheal disease cholera. This bacterium must endure many environmental stresses as it transits from an aquatic environment to the human gastrointestinal system. One of the mechanisms that are important in maintaining this infectious cycle is the production of the VPS. High expression of this PS is the cause of the rugose variant of an El Tor *V. cholerae* strain (66, 184). VPS was demonstrated to be essential for biofilm formation and resistance to chlorine. The NtrC family response regulator VpsR activates transcription of the *vps* operon, allowing for production of the PS (185).

The VPS is encoded by two operons, *vpsA* through *vpsK*, and *vpsL* through *vpsQ*. These operons are positively regulated by the response regulators VpsR and VpsT (185, 186). Expression analysis indicates that VpsR is epistatic to the positive regulator VpsT (187). Additionally, both VpsT and VpsR induce expression of each other, presenting a rapid positive feedback loop resulting in production of VPS (186). The negative regulator HapR represses VpsR, VpsT, and the *vps* operons. Collectively, these three regulators affect the expression of VPS and therefore biofilm phenotypes and colony rugosity. Deletion of *hapR* in a smooth strain relieves the negative regulation of *vps* expression, resulting in the transition to a rugose colony. Conversely, deletion of *vpsR* or *vpsT* in a rugose strain produces a smooth strain (187).

Several studies have indicated that *vps* genes are expressed during intestinal colonization and infection. VPS could aid in the formation of hyper-infectious aggregates of cells, as well as attachment and colonization of the intestine. In support of this, *vps* operon mutants demonstrated a significant defect in colonization of infant mouse intestines, supporting VPS being involved in host colonization (188).

Recent work indicates that the expression of the *vps* gene clusters is dependent on elevated intracellular levels of the second messenger c-di-GMP. VpsT binds c-di-GMP, resulting in oligomerization and upregulation of the *vps* operons (169, 170). Several diguanylate cyclases and phosphodiesterases affect VPS production by altering the cellular pool of c-di-GMP. Specifically, the phosphodiesterase VieA reduces *vps* expression by degrading c-di-GMP (189), while the diguanylate cyclases CdgA, H, K, L, and M all contribute to the activation of VpsT by producing elevated levels of c-di-GMP (170).

VPS consists mainly of glucose (52.6%) and galactose (37.0%), with small amounts of *N*-acetylglucosamine, mannose, and xylose (5.1%, 3.8%, and 1.5%, respectively). The primary linkages in this PS are 4-linked glucose and 4-linked galactose, suggesting that these sugars form the linear backbone. Branching of the PS is suggested by the detection of small amounts of 3,4- and 4,6-linked galactose and glucose, as well as 2,4-linked galactose (66).

Role in biofilm biology—Much of what is known regarding the role of VPS in biofilm biology stems from the observation of *V. cholerae* rugose variants. These colonies have a highly structured, wrinkled morphology due to the overproduction of VPS. When the VPS genes are deleted from a rugose strain, the colony reverts to a smooth morphology. In addition to the rough colony morphology, rugose strains demonstrate enhanced attachment and biofilm formation phenotypes (66, 185). Both *vps* operons are essential for VPS production and, therefore, biofilm development (188). Super-resolution confocal microscopy revealed that VPS promotes the retention of daughter cells in the biofilm, as well as the accumulation of the biofilm matrix proteins RbmA, Bap1, and RbmC (171). In the absence of VPS, biofilms were restricted to the monolayer stage. During biofilm growth, VPS was extruded from the cell and formed spherical foci on the cell surface. It was proposed that the VPS, along with the proteins Bap1 and RbmC, forms the biofilm matrix envelope around cell clusters. Collectively, VPS is regarded as an essential part of the *V. cholerae* biofilm matrix, which is important in both the environmental and host phases of the infectious cycle.

B. subtilis Polysaccharide

Significance, structure, and regulation—*B. subtilis* produces several PSs that affect biofilm structure and function, but the prevalent architectural PS is EPS (157, 190, 191). During a study of the formation of fruiting bodies, it was observed that Δ*yveQ (epsG)* and Δ*yveR (epsH)* mutants were unable to form structured pellicles or produce multicellular fruiting bodies (190). Sixteen genes encoding EPS are in an operon, and most are homologous to those involved in PS production and modification in other bacteria (190). A subsequent report updated the operon annotation to 15 genes and renamed it *epsA-O* (67). Further characterization of the genes involved in biofilm formation indicated that an unlinked gene, *ybxB*, is essential for biofilm formation (191). The Δ*ybxB* mutant forms pellicles that are similar to the EPS-deficient Δ*yveR* mutant. *yhxB* has sequence similarity to both phosphoglucomutase and phospho-mannomutase sugar-modifying enzymes that are involved in PS synthesis in other bacteria. It was concluded that *yhxB* along with the *epsA-O* operon are essential for the production of EPS and biofilms (191). Though this PS has been extensively studied, the structure is unknown (157).

The *epsA-O* operon is repressed by SinR (67), which binds to an operator upstream of the operon (67, 192). PS repression is relieved by SinI, which forms a complex with SinR and antagonizes its activity (67). As a result, SinR is deemed the master biofilm regulator of *B. subtilis* (67).

Role in biofilm biology—EPS is essential for the formation of pellicles and surface structures by *B. subtilis* (191). A Δ*epsH* mutant forms weak pellicles and smooth colonies, in contrast to the robust pellicle and rugose colonies formed by the wild type strain (67). Increased EPS expression in a Δ*sinR* mutant exhibits enhanced cell aggregation, pellicle formation, colony rugosity, and impaired swarming motility (67). The importance of PS in biofilm formation and cell aggregation is highlighted with the observation that addition of norspermidine can induce biofilm dissolution by targeting PS and interfering with its ability to aggregate and provide biofilm structure (193), though recent work has contradicted some of the claims about the effect of norspermidine (194), indicating that further research is required on this topic. Collectively, these data demonstrate the importance of the structural role of EPS in biofilm formation and stability.

Cellulose

Significance, structure, and regulation—Cellulose is one of the most abundant PSs in nature. The structure of cellulose fibrils is conserved throughout evolution, with bacterial cellulose indistinguishable from cellulose produced by higher-order algae, fungi, and plants. This polymer consists of repeating chains of β-1,4 linked D-glucose that form fibrils, which resemble cables (Fig. 1). Fibrils range from 1 to 25 nm in width and 1 to 9 μm in length and are assembled in the extracellular space immediately adjacent to the cell. These cables can align to form sheets, introducing the structural rigidity that plant cellulose provides due to its insoluble and inelastic nature. Cellulose fibrils have a tensile strength similar to steel (195). This structure is most evident in wood and paper products, which are largely composed of cellulose.

The role of cellulose in bacteria was first described in the Gram-negative bacterium *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) (196–198). This bacterium produces large quantities of highly pure cellulose that shares many properties with cellulose produced by higher-order organisms, which aided in research on the role of plant and algal cellulose. Static cultures of *G. xylinus* produce a robust pellicle that is rich in cellulose, facilitating the isolation of the polymer from the matrix. Subsequently, many bacteria have been found to produce cellulose, including commensal organisms such as *E. coli*, pathogens such as *S. enterica*, and environmental organisms such as many *Pseudomonas* spp. (199–201). The diversity in the niches of the organisms that produce cellulose highlights the variety of functions that it can play in the biofilm matrix.

The cellulose production genes have several names, depending on the species. These include *acs (Acetobacter* cellulose synthesis), *bcs* (bacterial cellulose synthesis), and *cel* (cellulose) (200). In general, the operon consists of two conserved genes and several accessory genes. The first conserved gene is *bcsA (acsA* or *celA*), which encodes the cellulose synthase enzyme. This is followed by *bcsB (acsB* or *celB*), which encodes a c-di-GMP binding protein (200). Additional genes in the operon can vary but generally include genes including a cellulase (*bcsZ* or *celC*) (200). The most thorough characterization of the cellulose biosynthetic machinery has been conducted in *G. xylinus*. In this bacterium, the cellulose synthase enzyme complexes are linked to the cytoplasmic membrane by 8 to 10 transmembrane domains. Approximately 50 synthase complexes form in a row along the long axis of the bacterium, excreting cellulose fibers that rapidly aggregate with nearby fibers to form a ribbon (200, 202).

Regulation of cellulose production is linked to c-di-GMP levels. In fact, the initial characterization of c-di-GMP and the diguanylate cyclase and phosphodiesterase enzymes responsible for this molecule were made in *G. xylinus* as a result of studies investigating factors that promote cellulose production (203, 204). c-di-GMP binds to BcsB and promotes the production of cellulose. In addition, cellulose production in *E. coli* and *S. enterica* is dependent on AdrA, which contains a GGDEF domain (199, 205). The cellulose synthase is expressed constitutively but is not active without the c-di-GMP inducer. It has been proposed that AdrA is the diguanylate cyclase that provides the c-di-GMP required for cellulose production to the cellulose synthase. This is supported by the evidence that expression of *adrA* from a plasmid is sufficient to induce cellulose production (199). This system provides rapid production of cellulose, since the biosynthetic machinery is present, requiring only production of AdrA to provide the allosteric inducer c-di-GMP.

Role in biofilm biology—Cellulose provides both structure and protection to bacterial cells in biofilms. The first reports of the contribution of cellulose to *G. xylinus* pellicles indicated that cellulose was responsible for cell aggregation in the pellicle, allowing the biofilm to float to the surface of the culture where oxygen was readily available to the bacteria (198). In addition to this role, cellulose provides protection from the mutagenic effects of ultraviolet light (195, 206).

The role of cellulose in biofilm formation has expanded across many species. One report describes the production of cellulose in many *Enterobacteriaceae*, including *E. coli,*

Salmonella enteritidis, Salmonella typbimurium, and *K. pneumoniae* (199). In these species, cellulose often interacts with the curli fimbriae to develop the biofilm matrix that is responsible for the *rdar* (rough, dry, and red) colony phenotype (199, 200). Other groups have observed that cellulose production results in pellicle formation in many *Pseudomonas* spp. (201). Cellulose production enhances binding of epithelial cells and reduces immune response to the bacteria in the probiotic strain of *E. coli* Nissle 1917 (207). These functions are important for establishing a commensal relationship with the host epithelium. Cellulose is produced by many bacteria to utilize the properties of this simple polymer for a wide variety of functions, providing a good example of the functional diversity of PSs.

FUTURE PERSPECTIVES

A wealth of knowledge has been gained regarding the composition and functions of PS components of the biofilm matrix by utilizing methods such as traditional genetic techniques combined with biochemistry and immunochemistry, lectin/carbohydrate stains, and microscopy (208). While necessary for initial observations, this reductionist point of view leaves significant gaps in our understanding of biofilm biology. PS isolation is difficult to achieve, especially from environmental or host sources, which contain a diverse range of components. Moreover, the paucity of animal models, particularly for chronic infections, presents a significant challenge for correlating *in vitro* findings to infection. Many bacteria produce multiple PSs, and *in vivo* infections are rarely comprised of a single microbe. Our current understanding of how PSs interact in polymicrobial communities in the environment or the host is extremely limited. Bridging this gap will require the development of more extensive *in situ* techniques to visualize, measure, and fully define PSs in real-world situations. Further development and utilization of microscopy techniques such as super-resolution and Raman scattering microscopy, combined with fully hydrated living models, will be essential to provide a complete interpretation of the complexity of microbial biofilms and the contribution of PS production (209).

While PSs are a predominant and well-studied biofilm component, the matrix is clearly composed of a range of macromolecules, including eDNA, proteins, and lipids. It stands to reason and is frequently speculated that these molecules interact in the matrix to form specific and dynamic interactions to fine-tune the biofilm community to varying environments. However, information regarding the biological significance, regulation, and direct visualization of these interactions are currently lacking. For example, since the seminal discovery by Whitchurch and colleagues in 2002 that eDNA is an important component of *P. aeruginosa* biofilms (210), only a few studies have defined how eDNA interacts with PS in the matrix. It has been suggested that in *Myxococcus xanthus* biofilms, eDNA directly interacts with PS, enhancing the physical strength and resistance to biological stress (211). Accordingly, a mathematical model of biofilm stress relaxation reveals evidence that filamentous eDNA may interact with PS to produce well-defined agglomerate structures with unique physioelastic properties (212). For protein-EPS interactions, an additional observation in *M. xanthus* illustrated the first direct interaction of EPS and type IV pili (213). However, the biological significance and the nature of these interactions remain unclear, representing a significant gap in our understanding of the biofilm matrix.

In addition to interacting with other components of the biofilm matrix, it is likely that various PSs, produced by either the same bacteria or by other species in the environment, interact with each other to produce unique and/or compensatory functions. Some PSs may be exclusively expressed at specific stages of biofilm formation or under certain environmental conditions. Interrogating the spatial and temporal production of PSs and how they interact with other molecules in the environment will significantly enhance our understanding of how biofilms develop and how they may be modulated.

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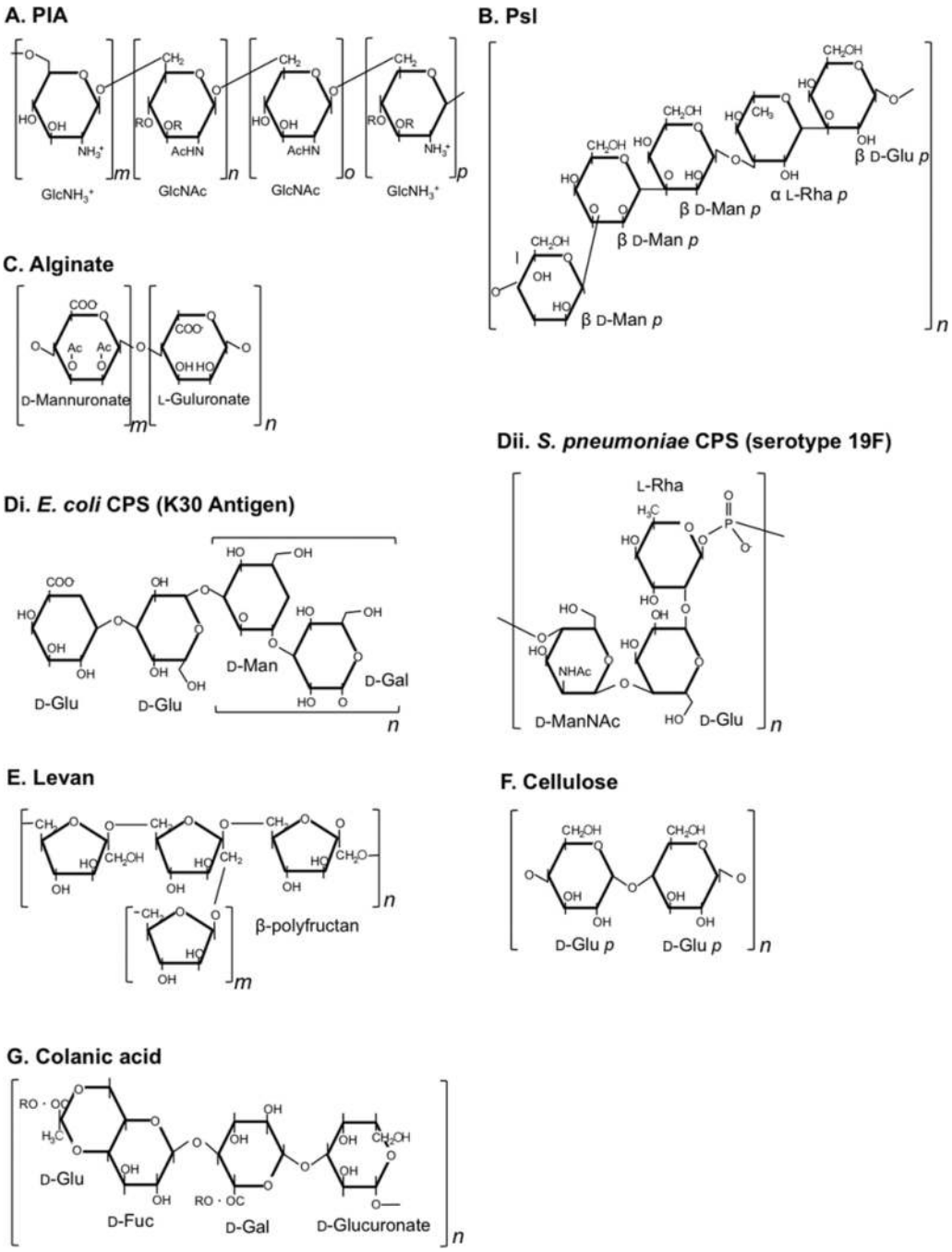

FIGURE 1.

Adapted representative chemical structures of polysaccharides which participate in biofilm formation including (A) polysaccharide intercellular adhesin (PIA), (B) Psl, (C) alginate, capsular polysaccharide (CPS) from (Di) *E. coli* and (Dii) *S. pneumoniae*, (E) levan, (F) cellulose, and (G) colanic acid. Brackets depict repeating units. doi:10.1128/microbiolspec.MB-0011-2014.f1

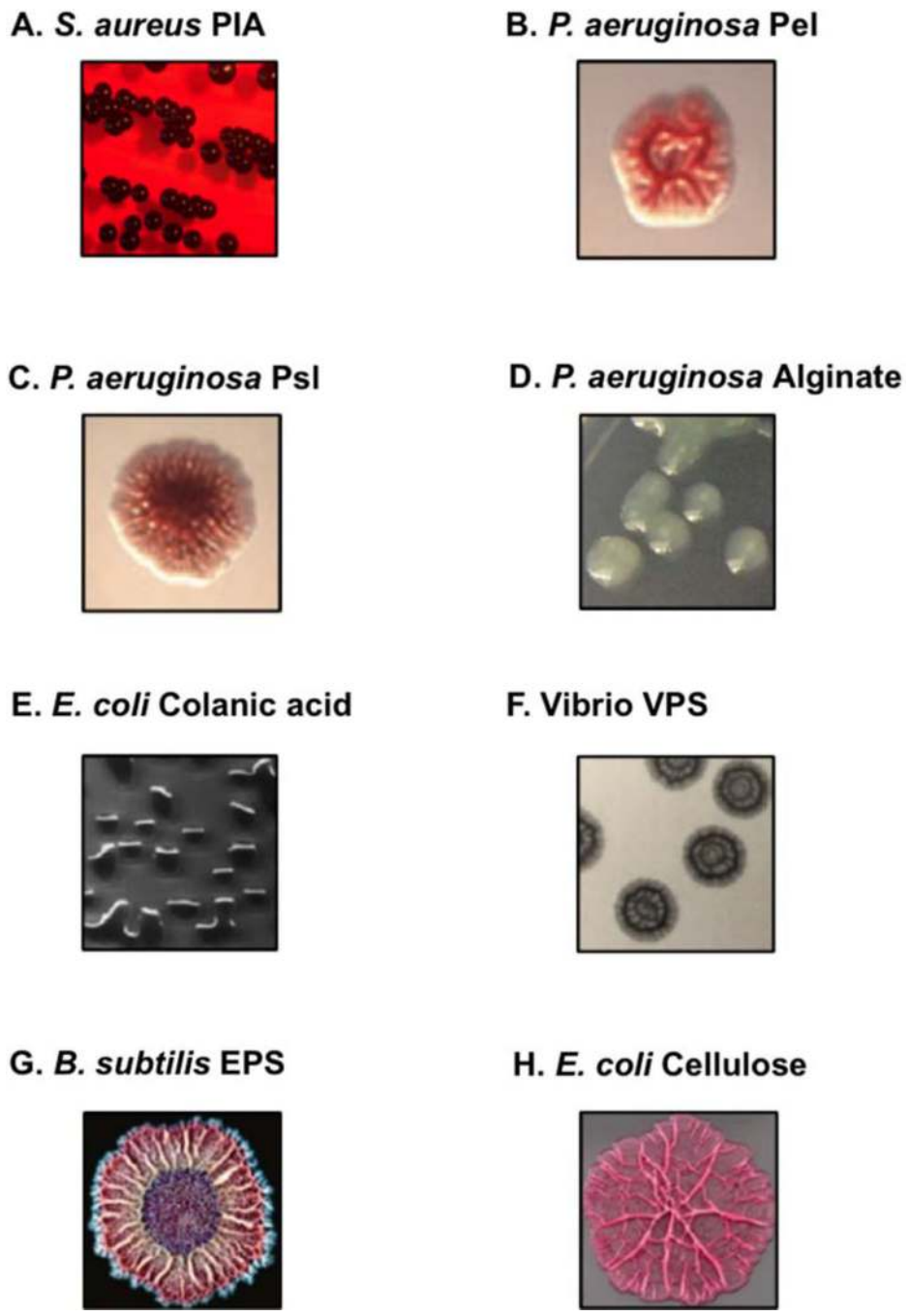

FIGURE 2.

Colony phenotypes conferred upon expression or overexpression of PS by representative bacteria. (A) PS intercellular adhesion producing *Staphylococcus aureus*. Reprinted from *World Journal of Microbiology and Biotechnology* (214) with permission from the publisher. (B) Pel producing *Pseudomonas aeruginosa* (Δ*wspF*Δ*psl*). Reprinted from *Molecular Microbiology* (215) with permission from the publisher. (C) Psl producing *P. aeruginosa* (Δ*wspF*Δ*pel*). Reprinted from *Molecular Microbiology* (215) with permission from the publisher. (D) Alginate overproducing *P. aeruginosa (mucA22)*. Not previously

published. Credit: Daniel Wozniak. (E) Colanic acid producing *Escherichia coli*. Reprinted from *PLoS One* (216) with permission from the publisher. (F) VPS producing rugose variant of *Vibrio cholerae*. Reprinted from *The Proceedings of the National Academy of Sciences of the USA* (66) with permission from the publisher. (G) EPS producing *Bacillus subtilis*. Reprinted from *The Proceedings of the National Academy of Sciences of the USA* (217) with permission from the publisher. (H) Cellulose producing *E. coli* (*csgD::cm*). Reprinted from *The Journal of Medical Microbiology* (218) with permission from the publisher. doi: 10.1128/microbiolspec.MB-0011-2014.f2

TABLE 1

Summary of the cellular location, chemical composition, and functions of bacterial polysaccharides important for biofilm formation

			Functions		
	Localization	**Charge**	**Aggregative**	**Protective**	**Architectural**
Pel	Secreted	NA	X	X	X
Psl	Secreted/cell associated	Neutral	X	X	X
PIA	Secreted	Polycationic	X		X
Cellulose	Secreted	Neutral	X	X	
Alginate	Cell associated	Polyanionic		X	X
CPS	Covalently attached	Polyanionic		X	
Levan	Cell associated	Neutral	X	X	
Colanic acid	Cell associated	Polyanionic			X
VPS	Secreted	NA	X	X	X
***Bacillus* EPS**	Secreted	Neutral			X