

What's on the Outside Matters: The Role of the Extracellular Polymeric Substance of Gram-negative Biofilms in Evading Host Immunity and as a Target for Therapeutic Intervention*

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Biofilms are organized multicellular communities encased in an extracellular polymeric substance (EPS). Biofilm-resident bacteria resist immunity and antimicrobials. The EPS provides structural stability and presents a barrier; however, a complete understanding of how EPS structure relates to biological function is lacking. This review focuses on the EPS of three Gram-negative pathogens: *Pseudomonas aeruginosa*, nontypeable *Haemophilus influenzae*, and *Salmonella enterica* serovar Typhi/Typhimurium. Although EPS proteins and polysaccharides are diverse, common constituents include extracellular DNA, DNABII (DNA binding and bending) proteins, pili, flagella, and outer membrane vesicles. The EPS biochemistry promotes recalcitrance and informs the design of therapies to reduce or eliminate biofilm burden.

Biofilms, which are defined as highly organized multicellular communities of bacteria encased in an extracellular polymeric substance (or EPS),³ contribute to most chronic infections in the body. These infections confer a significant socioeconomic burden with treatments costing billions of dollars annually. Biofilm formation is key to the establishment of most chronic bacterial infections, and a majority of the population will expe-

rience some form of chronic or recurrent bacterial disease in their lifetime. Bacteria resident within a biofilm are highly resistant to phagocytosis and other clearance mechanisms and demonstrate substantially increased resistance to immune effectors and antibiotics (see the accompanying review by van Acker and Coenye (1)). Much of this resistance can be attributed to the EPS, which presents a formidable physical barrier to cellular effectors of immunity and is highly recalcitrant to removal. Although the EPS can be diverse, depending on the microbe(s) that initiate its formation, most are composed of bacterial proteins, polysaccharides, and extracellular DNA (eDNA) (2). Moreover, as the EPS matrix encases the microbes, it is the first to interact with the human immune system. Although immune-mediated clearance of biofilms is often ineffective, the mechanistic basis is as yet undefined (3–5).

To develop new therapeutic strategies to limit chronic human infection, it is crucial to understand how the biofilm EPS provides protection against antimicrobials and the immune system. Addressing this requires a greater understanding of biofilm maturation *in vivo* with a focus on discerning what factors influence whether the immune response either augments biofilm formation, contributing to persistence, or results in effective biofilm disruption with clearance of the pathogen. This would include where, when, and why the immune system fails to mediate clearance and/or actually facilitates maintenance of long-term biofilms and carriage. The overall goal of this review is to describe the interface between host immunity and the biofilm EPS matrix of three important and well studied human pathogens (*Pseudomonas aeruginosa* (*Pa*), nontypeable *Haemophilus influenzae* (NTHI), and *Salmonella enterica* serovar Typhimurium/Typhi (*St/Sty*)). The human diseases under investigation in this proposal include airway and other chronic infections (wounds, gastrointestinal disease, and otitis media (OM)). Sophisticated, well established animal models to both study each of these infections and conduct pre-clinical evaluation of biofilm-focused therapeutic modalities will also be discussed.

EPS Components

Bacteria within biofilms are usually embedded in a matrix, which consists of protein, polysaccharide, and nucleic acid, and the matrix provides a critical role in the biofilm resistance phenotype (2, 6). In this section, a brief description of the major *Pa*, NTHI, and *St/Sty* EPS components will be described (Table 1) to allow readers a better understanding of their roles in biofilm biology, topics discussed later in this review.

Exopolysaccharides

Exopolysaccharides contribute to the biofilm matrix of all three organisms discussed herein. Perhaps the best studied is *Pa*, which encodes at least three distinct polymers (Psl, Pel, and alginate). The composition, role, and regulation of each of these matrix components have been the subject of several recent reviews (7, 8). Expression of alginate confers a mucoid phenotype to the organism, seen most often in *Pa* isolated from later

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³ The abbreviations used are: EPS, extracellular polymeric substance; DNABII, DNA binding and bending; eDNA, extracellular DNA; HU, histone-like protein; *Pa*, *Pseudomonas aeruginosa*; NTHI, nontypeable *Haemophilus influenzae*; *St/Sty*, *Salmonella enterica* serovar Typhimurium/Typhi; OM, otitis media; CF, cystic fibrosis; LOS, lipooligosaccharide; AMP, antimicrobial peptide; OMV, outer membrane vesicle; IHF, integration host factor; CDA, *cis*-2-decenoic acid; QS, quorum sensing; DPD, 4,5-dihydroxy-2,3-pentanedione.

TABLE 1
EPS components of bacterial biofilms

	Bacteria and components			References
	<i>Pa</i>	NTHI	<i>St/Sty</i>	
Polysaccharide				
Psl	LOS		O-antigen capsule Cellulose Colanic acid ^a Vi-Antigen ^b	7, 8, 70, 81, 101, 102
Pel				
Alginate				
Protein				
CdrA	18 biofilm-specific proteins including major OMPs ^c P1, P2, and P5		Curli (amyloids) BapA	19, 20, 26, 27, 103
LecA/LecB DNABII proteins (IHF and HU)	DNABII proteins (IHF and HU)		DNABII proteins (IHF and HU)	
eDNA				
Yes	Yes		Yes	6, 17
Others				
Outer membrane vesicles Type IV pili Amyloid (Fap)	Outer membrane vesicles Type IV pili		Flagella	82, 12, 17, 25, 104

^a Produced in non-typhoidal serovars, but not in serovar Typhi.

^b Produced by typhoidal strains (serovars Typhi, Paratyphi C) as well as serovar Dublin, but not other non-typhoidal serovars.

^c OMPs, outer membrane proteins.

stages of cystic fibrosis (CF) lung infections. Psl and Pel are expressed in most non-mucoid *Pa* strains. Released Psl is a neutral charged polymeric pentasaccharide, yet the structure of the cell-associated Psl is not known (9, 10). Pel is a positively charged polymer (11). Each of these polysaccharides has distinct roles in biofilms formed by mucoid or non-mucoid *Pa* strains (7). In NTHI, there has yet to be an exopolysaccharide identified that clearly contributes to biofilms, yet the lipooligosaccharide (LOS) plays a prominent role in modulating biofilm structure. NTHI LOS can be modified by the addition of phosphorylcholine, although the role of this in biofilm function is not clear (12, 13). The composition of the *Salmonella* spp. biofilm matrix is complex and highly variable in response to altered environmental conditions and variable among serovars. The *St/Sty* polysaccharides identified to date include colanic acid, O-antigen capsule, and cellulose (14, 15). *Sty* produces a distinct polysaccharide, Vi-antigen, a primary marker for infection with this serovar and the basis of several current vaccine approaches.

eDNA

Another abundant biofilm matrix building block, eDNA, is a critical component of the NTHI, *Pa*, and *St/Sty* biofilm matrix (6, 16, 17). The source of eDNA appears to be random genomic sequences of varying lengths, with no apparent sequence selectivity. eDNA is apparently derived from stochastic lysis of a subpopulation of the bacteria within the biofilm. In most situations, eDNA is a contributing component of a more diverse biofilm matrix and typically interacts with proteins or polysaccharides to stabilize the matrix. In *Pa* and *St*, eDNA binds Pel polysaccharide or amyloid fibers, respectively (11, 18). Interactions of eDNA with other biofilm matrix components likely explain why DNase treatment of biofilms has variable effects on destabilizing the matrix.

Proteins

Proteins are increasingly recognized for their importance in biofilm structure and function. For many organisms, the structure and resistance properties of biofilms can be eliminated by

protease treatment (19, 20). Matrix proteins include secreted proteins as well as components of adhesins or motility organelles. Large-scale proteomic studies have been performed on EPS recovered from both NTHI and *Pa* (20, 21). Consistent from these studies and others is the abundance of outer membrane proteins and type IV pili in these matrix preparations. The best studied matrix proteins produced by *Pa* include lectins LecA and LecB (22) and CdrA (23). Although both LecA and LecB are carbohydrate-binding proteins involved in biofilm formation, it is not clear how they contribute to matrix formation and neither appears to associate with Pel or Psl. CdrA, a large extracellular adhesin-like protein, associates with Psl and contributes to biofilm integrity (23). For NTHI, one of the best studied matrix proteins is type IV pilin protein, which has been shown to: 1) serve as a constituent of the EPS; 2) be necessary for twitching motility; and 3) contribute significantly to the architecture of an NTHI biofilm (17). The main proteinaceous component of *St* biofilms is curli pili, which structurally and biochemically are amyloid fibers. Curli appear to promote bacteria-surface and bacteria-bacteria interactions that enhance biofilm stability (24). Additionally, *St* surface flagella mediate attachment to cholesterol (primary constituent of gallstones) in the initial stages of biofilm development, whereas fimbriae do not appear to play a significant role in this process (25). Also, BapA is a large surface protein variably associated with the production of robust biofilm formation in *Salmonella* spp. (26). Finally, the DNABII family of proteins (e.g. HU and IHF) has been observed associated in a highly organized fashion with the eDNA outside of the bacterium (27) (see below).

Outer Membrane Vesicles (OMVs)

Most Gram-negative bacteria produce OMVs that contain a diverse array of molecules, which contribute to a variety of biological processes. Bacterial OMVs can allow trafficking of biomolecules to other cells in their environment. Studies showed that OMVs are definitive components of the *Pa* and NTHI biofilm EPS (28, 29), and they contain cargo that may contribute to the matrix EPS (Table 1). Although mutants that regulate both *Escherichia coli* and NTHI OMV biogenesis have

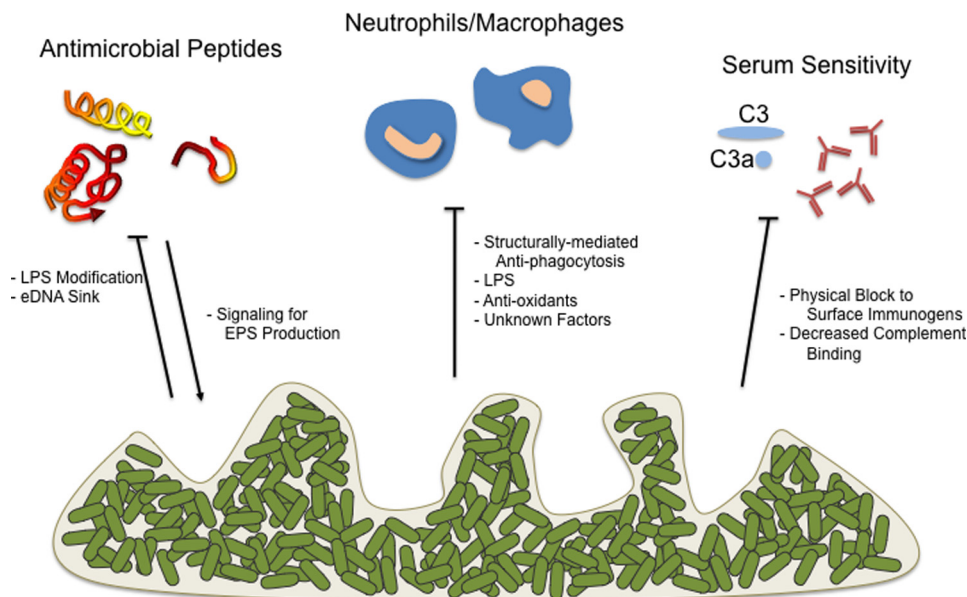


FIGURE 1. Role of EPS in promoting recalcitrance to host immunity. The biofilm architecture, as well as products released by biofilms, can help mediate resistance to immune clearance. Categories of factors to which biofilms provide recalcitrance include, in addition to traditional antibiotics, antimicrobial peptides, engulfment/killing by professional phagocytes, and opsonization (antibodies/serum complement). Additionally, in some instances, host inflammation can signal biofilm EPS production (arrow). Horizontal line termini denote biofilm-mediated resistance to the immune effector.

recently been identified (30), their precise role in modulating biofilm/EPS structure has not been evaluated.

Role of EPS in Promoting Recalcitrance to Host Immunity

The biofilm EPS of *Pa*, NTHI, and *St/Sty* promote resistance to killing by innate immune constituents (Fig. 1) including antimicrobial peptides (AMPs), professional phagocytes, and serum factors. Part of this resistance is mediated by the biofilm community structure, which likely sterically limits engulfment by host cells, as well as the penetration of immune components. The eDNA and polysaccharides can bind and sequester immune components, particularly those with a charge differential such as AMPs and matrix eDNA. Opsonization by complement and by immunoglobulins is also negatively affected by EPS. The bacteria within the biofilm respond by producing factors that limit the oxidative and non-oxidative capabilities of phagocytic cells, aiding bacterial survival. Additionally, as exemplified by *Pa*, host immune components can promote *Pa* diversification with variants having enhanced EPS production providing further recalcitrance (31, 32).

It is well accepted that the biofilm mode of growth affords *Pa* protection from host immune effectors (33). This appears to be independent of where *Pa* biofilms form in its host. One of the first studies to address this revealed that human neutrophils are capable of penetrating biofilms and carry out phagocytosis and granule secretion. However, the neutrophils exhibit a distinct non-reactive morphology and increase oxygen consumption, yet fail to kill bacteria (4). The precise mechanisms underlying this mitigation of neutrophil defense function is currently unknown. Perhaps the best recognized recalcitrance mechanism is the production of quorum sensing-dependent rhamnolipid by biofilm-grown *Pa*. Rhamnolipid has potent cytotoxicity toward polymorphonuclear leukocytes (3). As with several other exopolysaccharides, the presence of Psl or alginate on the

Pa surface inhibits phagocytosis by limiting opsonin deposition (34, 35). Alginate also provides *Pa* protection from IFN- γ -mediated macrophage killing (36). Expression of both Pel and Psl in *Pa* affords protection against clearance in murine models of acute infection (35, 37). Likewise, eDNA provides resistance to AMPs and aminoglycosides by chelating cations, which otherwise could lead to perturbation of *Pa* membrane structure/function and induction of the PhoPQ and PmrAB regulon. Positively charged AMPs can bind eDNA, sequestering them from the bacterial cell surface (38). Of note, *Pa* variants that emerge during chronic infection have a hyper-biofilm phenotype and express elevated levels of EPS, which provides these bacteria further protection from host defenses. Often, products of the robust inflammatory response (e.g. H₂O₂ or antimicrobial peptides) enhance the frequency of variants that emerge (31, 32, 39).

The NTHI EPS also confers immune resistance to the resident microbes via a variety of mechanisms. One of the first examples was that LOS provided resistance to phagocytosis by polymorphonuclear leukocytes *in vitro* as well as to extracellular killing mediated by the action of histones and elastase within neutrophil extracellular traps (40). Izano *et al.* (41) utilized both proteinase K and DNase treatment to measure resistance of NTHI biofilms to a variety of biocides. They concluded that the cohesive properties conferred by proteinaceous intercellular adhesins and the eDNA within the EPS contributed to resistance, perhaps by providing a barrier to penetration and/or by sequestration of these agents within the matrix. A recent study (42) demonstrated that NTHI-produced peroxiredoxin-glutaredoxin and catalase promote resistance to oxidants and survival within neutrophil extracellular traps, whereas another study showed that production of the DNA-binding protein Dps also confers resistance of NTHI to oxidative stress *in vitro* and

to clearance *in vivo* (43). Finally, in addition to providing structure to NTHI biofilms, eDNA present within the biofilm matrix binds the AMP human β -defensin 3, thus limiting its access to bacteria resident within the biofilm and reducing its antimicrobial activity (44).

Salmonella species produce numerous EPS components *in vitro*; however, comparatively little is known about the *in vivo* condition. These EPS components include cellulose, colanic acid, O-antigen capsule, Vi antigen, curli pili, eDNA, and other protein components. Some of these EPS members are species-specific: for example, colanic acid (produced only in non-typhoidal species) and Vi-antigen (produced in typhoidal species). Winter and colleagues (45, 46) showed that the *Sty* Vi-antigen prevents complement receptor 3 (CR3)-mediated clearance and neutrophil chemotaxis, and results in reduced IL-8 production. O-antigen capsule and Vi capsular polysaccharide prevent complement-mediated clearance of *Salmonella* (45, 47). Deletion of colanic acid genes enhances antibody production to vaccine strains, likely by unmasking surface antigens (47). Curli pili, on the other hand, are sensed by Toll-like receptor (TLR) 1/2 and NLRP3 (48, 49). Additionally, the two-component regulatory systems PhoPQ and PmrAB mediate AMP resistance in *Salmonella* in part by divalent cation sensing via a periplasmic acidic patch of PhoQ and induction of LPS modification. The production of eDNA is a key component of *Salmonella* biofilms, and the chelation of Mg^{2+} in biofilm eDNA mediates AMP resistance in a PmrAB/PhoPQ-dependent manner (16).

Animal Models to Interrogate EPS Biofilm Attributes

Experiments in animals are often essential for defining the virulence potential of microbes and the host response to infection, and validating treatment modalities. The development of models that faithfully mimic human chronic biofilm diseases has been challenging. Striking an appropriate balance between modeling the persistent disease state (biofilm) yet limiting overt systemic spread typically requires careful attention to the pathogen burden, providing localized, confined delivery, and monitoring/manipulation of the immune system. Such methods have been applied to the development of chronic infections involving *Pa*, NTHI, or *Sty*. The advantages and potential limitations of each method are discussed.

Pa causes an array of both chronic and acute infections (50). As such, there are several models that have been developed to mimic such infections (for a review, see Ref. 51). Although most models provide insights into the pathogenesis of acute disease, there are often issues with the chronic models. In fact, progress in our understanding of the role of biofilms in persistence and the development/testing of therapeutics in many *Pa* chronic infections is hampered by the absence of suitable models. Nonetheless, murine models do exist for the study of CF airway diseases. Perhaps the most widely used is the intratracheal instillation in rodents of *Pa* embedded in either agar or alginate beads (52). The agar or alginate matrix provides protection against clearance. Although variations of this have also been adapted to *cftr*-defective mice, these models do not exhibit characteristic CF ion transporter defects in the lungs and fail to faithfully reproduce the chronic infections that occur in

humans. More promising CF models include the epithelial sodium channel (ENaC)-overexpressing mouse (53) and the porcine CF model (54). Perhaps the best systems to study chronic *Pa* infections lie in the various wound models that have been developed. These incorporate murine systems, including those with chronic diabetes syndrome (55, 56). As wound healing dynamics and pathogenesis in murine systems differ from those in humans, others have implemented porcine wound models of persistent infections with *Pa* growing as single or mixed species biofilms (57, 58). Other biofilm-associated models, including those that mimic device related/implant or corneal infections, have also been applied with success (50, 51, 59).

NTHI induces multiple diseases of the upper and lower respiratory tract including OM, sinusitis, bronchitis, and exacerbations of both chronic obstructive pulmonary disease (COPD) and CF. The majority of these include a biofilm component, which contributes greatly to the chronicity and/or recurrence of disease. To model these diseases and investigate the role of biofilms in pathogenesis, two rodent hosts have been relied upon. For OM, the chinchilla has served as the predominant mammalian host (60). This has become a robust and highly reproducible model of this prevalent pediatric disease (61) with the formation of large biofilms that remain in the middle ear for many weeks. In the majority of these studies, NTHI was inoculated directly into the middle ear space wherein biofilms form rapidly; however, in chinchillas that have been co-challenged with respiratory syncytial virus (RSV), NTHI, and *Moraxella catarrhalis*, both *M. catarrhalis* and NTHI ascend the Eustachian tube to induce mixed species biofilm formation in the middle ear (62). The murine host has served as the predominant rodent model for demonstration of biofilm formation by NTHI in the lower airway and investigation of its role in chronic obstructive pulmonary disease and other pulmonary infections (43, 63); however, the significantly smaller size of this host has limited the biomass of samples available for evaluation.

Salmonella colonize humans and animals to cause a spectrum of diseases, with the primary clinical manifestations of gastroenteritis and typhoid fever. *Sty* is the primary etiologic agent of typhoid fever, which is an acute illness, but can result in a chronic, asymptomatic infection primarily localized to the gallbladder (14). Gallbladder colonization by *Salmonella* during chronic infection has been known for more than a century. A high percentage of human carriers harbor gallstones, and biofilms form on gallstone surfaces during chronic carriage (64, 65). A mouse model of carriage has been developed based on the documented long-term survival of *St* (the mouse model for the human-specific *Sty*) in NRAMP1^{+/+} (SLC11A1) mice (129X1/SvJ) and the ability to induce gallstone formation in the mouse with a lithogenic diet (66). Such gallstone-containing mice harbor a 5000-fold increase in bacteria in the gallbladder and demonstrate a 500-fold increased shedding in feces, while bacterial biofilms can be observed on gallstone surfaces. Studies have been carried out to 1 year after infection in the 129X1/SvJ model, showing colonization of the gallbladder, as well as distal sites (mesenteric lymph nodes and bone marrow) (67, 68). Biofilm formation on the gallbladder epithelium and epithelial cell invasion have also been observed in the chronic mouse model

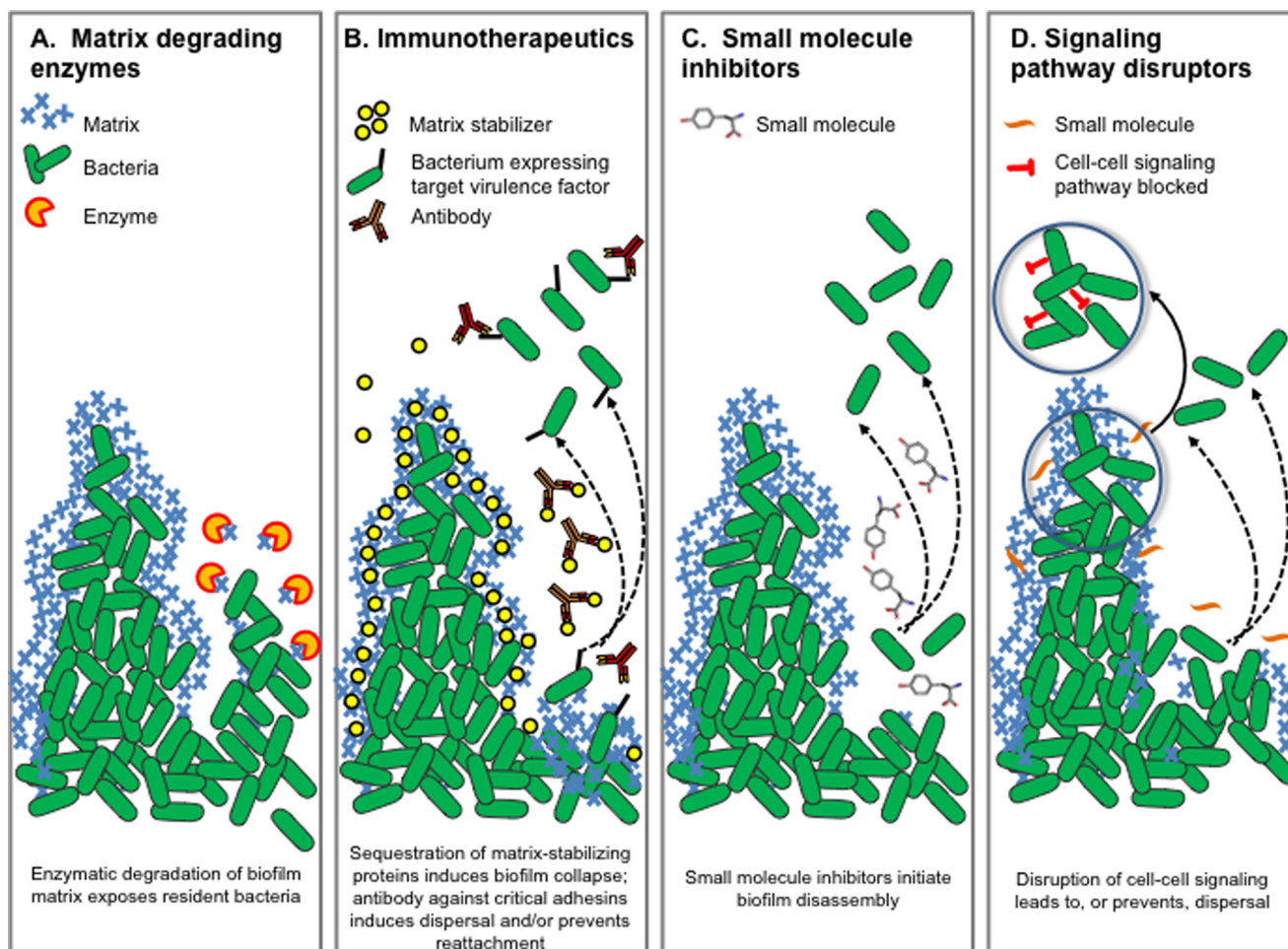


FIGURE 2. Therapies that target the EPS to reduce or eliminate biofilm burden. Four therapeutic strategies targeting EPS (A–D) are indicated and discussed further in the text. Symbols depicting methods and targets are indicated along the top, and proposed mechanisms of action are indicated at the bottom of each panel.

and may account for persistence in the absence of gallstones (67). In human carriers and in acute mouse models, gallbladder tissues possess *Salmonella* within and/or on the epithelium (69). Explanted tissue from the chicken intestinal epithelium has also been used to study *Salmonella* biofilms and intestinal colonization (70).

Therapies Targeting EPS to Reduce or Eliminate Biofilm Burden

Diseases wherein a biofilm contributes to the chronic and recurrent nature of the disease course require novel methods for diagnosis, treatment, and prevention. Given the recalcitrant nature of biofilm-resident bacteria to the action of antibiotics, a variety of non-antibiotic approaches are being investigated (Fig. 2), including those that focus on physical disruption, surgical removal, and even *ex vivo* thermal mitigation to eradicate biofilms present on implanted medical devices (71). This area of research is beyond the scope of this article; however, there have been several recent excellent reviews (72, 73). The following strategies have been explored for their ability to disrupt established *Pa*, NTHI, and *St/Sty* biofilms, either *in vitro* or *in vivo*.

Matrix-degrading Enzymes

Given that eDNA is a common EPS constituent, treatment of biofilms with DNase has been explored as a mechanism for biofilm disruption for many microbes including NTHI (74) (Fig. 2A). In certain diseases, disruption of the heavy DNA strands contributed by neutrophil netting (e.g. as in the viscous middle effusion recovered from children with chronic OM) by DNase provides an additional desired clinical outcome. Given the abundance of alginate in biofilms produced by many mucoid *Pa* isolates, the use of alginate lyase to disperse biofilms has been investigated (75). A recent study (76) reinforced the conclusions about the biofilm-disruptive properties of this approach as well as its synergistic interaction with antibiotics. However, they present data that question the mechanisms that underlie alginate lyase enzyme-based therapies. Another approach for disruption of biofilms formed by *Pa* has involved the use of glycoside hydrolases to target exopolysaccharides present within the EPS, as the enzymes PelA_H, PslG_H, and Sph3_H can disrupt existing *Pa* biofilms *in vitro* (77, 78). The accompanying review by Sheppard and Howell discusses this class of enzymes in more detail (79). Likewise, the enzyme Dispersin B in combination with an AMP showed synergistic antibiofilm/

antibacterial activity in a chronic wound model of *Pa* infection (80). For *St*, cellulase has been used *in vitro* to target cellulose, which often (depending on growth conditions) has a dramatic negative effect on biofilm formation (81). DNase is also highly effective at disrupting eDNA-rich biofilms formed by *St* (18).

Immunotherapeutics

Constituents of the biofilm EPS can also serve as targets for immune intervention as a result of either a natural immune response or one directed by immunization (Fig. 2B). Biofilms already established in the middle ears of chinchillas were resolved following transcutaneous immunization with a chimeric immunogen that incorporated epitopes of type IV pili and outer membrane protein P5, delivered with the adjuvant dmLT, a double mutant of the *E. coli* heat labile enterotoxin (83). Clearance of these established biofilms was attributed to the action of immunogen-specific IgG and IFN γ - and IL-17-producing CD4⁺ T-cells and secretion of host defense peptides within the middle ear (84). Within this same line of investigation, by targeting a lynchpin protein that is positioned at the vertices of crossed strands of eDNA present within the biofilm matrix (e.g. either IHF or HU of the DNABII family of DNA-binding proteins), biofilms formed by NTHI, *St/Sty*, and *Pa* can be significantly disrupted when exposed to antiserum directed against a DNABII protein. This treatment is proposed to induce an equilibrium shift, which removes these proteins from the biofilm matrix, thereby mediating catastrophic structural collapse (27).

Small Molecule Inhibitors

In an early study, the ability of a mixture of D-amino acids to prevent *Pa* biofilm formation (85) suggested that they might similarly be useful to disrupt these biofilms (Fig. 2C). Others found that specific D-amino acids disrupted *Pa* biofilms and were particularly effective when combined with antibiotics (86). When used alone, however, treatment of *Pa* biofilms with a mixture of D-amino acids induced ~30% increase in matrix production, thereby suggesting the potential to inadvertently provide protection for any remaining viable or persister cells (86). Recently, Leiman *et al.* (87) indicated that D-tyrosine actually inhibited bacterial growth, and others have not found D-amino acids to be effective (88). Additional studies are clearly needed to resolve the exact mechanism of action. Much of the effort with *St* biofilm disruption has focused on chemical disruption to mediate sterilization of food processing surfaces (not reviewed here), but a broad-spectrum anti-biofilm peptide (peptide 1018) was utilized to induce a disruptive cellular stress response in *St*, *Pa*, and other bacteria (89). This peptide eradicated mature biofilms when used at low concentrations by targeting the enzymes RelA and SpoT, which mediate the synthesis of two small signaling nucleotides (collectively referred to as guanosine tetra- or pentaphosphate guanosine ((p)ppGpp)) involved in the stringent response. Although little additional literature exists regarding *St/Sty* dispersal agents, an ATP-mimetic biofilm inhibitor compound that reduced initial *St* binding but did not disrupt existing biofilms has been identified (90). This compound also showed activity against *Acinetobacter baumannii*. Furthermore, several different classes of potent *St*

and *Pa* biofilm inhibitors have been identified, centered on brominated furanones, 2-aminoimidazoles, and 2-aminoimidazole-based compounds (91, 92).

Signaling Pathway Targets

Many signaling pathways have been implicated in biofilm development, including two-component regulators, cyclic nucleotides, nitric oxide, phenazines, small peptides, and quorum sensing (QS) (Fig. 2D). Due to its pivotal importance in biofilm development, much of the work in this area has focused on the action of the second messenger cyclic di-GMP (see the accompanying background in the Valentini and Filloux review (93)). A recent study showed that the diguanylate cyclase GcbA mediated *Pa* biofilm dispersal via activation of the chemosensory protein BdlA (94). In a related line of investigation, a substituted fatty acid messenger, *cis*-2-decenoic acid (CDA), produced by *Pa* can disperse biofilms formed by a range of bacteria and even *Candida albicans* (95). A recent study (96) using a microarray-based approach to better define the CDA-mediated signaling pathways and mechanisms involved identified enhanced motility, altered metabolic activity, virulence, and persistence at varied temperatures. Use of CDA in combination with antimicrobials mediated the best results. Although characterization of the QS pathways utilized by NTHI, as well as defining their important role in pathobiology, is an area of active investigation, to date this approach has not been extensively explored as a means to disrupt existing biofilms despite recognition of its potential (97). One recent *in vivo* study, however, showed that the ability to disrupt pre-existing NTHI biofilms in the middle ears of chinchillas following immunization with the majority subunit of the type IV pilus was dependent upon the production of AI-2 quorum signaling molecules via the activity of the 4,5-dihydroxy-2,3-pentanedione (DPD) synthase, LuxS (98). This suggests that immune pressure could induce a dispersal response. Moreover, the addition of DPD to type IV pili⁺ NTHI biofilms mediated their dispersal. Although comparatively less is appreciated regarding QS signaling and biofilm development in *St*, several investigators have considered the feasibility of this approach, and although not tested for their ability to disrupt an existing biofilm, an alkyl-DPD panel of AI-2 inhibitors potently inhibits *St* QS (99). The accompanying review by Kavanaugh and Horswill (100) provides an excellent discussion of the *Staphylococcus aureus agr* QS pathway, which controls the production of exotoxins and exoenzymes required for infection and biofilm production and dispersal.

Synopsis and Perspectives

The ability of a bacterium to form a biofilm aids the establishment and development of recurrent and chronic infection. Moving forward, challenges include the development of animal models that accurately model human infection, the definition of EPS components produced *in vivo* and their relative contributions to the establishment and maintenance of chronic infection, anti-biofilm discovery with *in vivo* efficacy, and a more complete understanding of immune modulation by biofilm components. Much remains to be learned regarding persistent bacterial infections and immune system interactions, but the

study of *Pa*, NTHI, and *St/Sty* biofilms *in vitro* and *in vivo* has helped to advance this work.

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