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LEADING ARTICLE

A Transition to Targeted or 'Smart' Vaccines: How Understanding Commensal Colonization Can Lead to Selective Vaccination

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Abstract Bacteria represent a group of biological organisms responsible for causing a myriad of human illnesses such as bacteremia, pneumonia, meningitis, and skin infections. Although there have been substantial improvements in treating bacterial diseases, addressing diseases associated with the formation of physiological communities called biofilms remains a challenge. This is due to the complexity of biofilms, both structurally and phenotypically, which complicates the development of comprehensive prophylactic and therapeutic interventions. This situation is exacerbated by the inability of current in vitro and in vivo biofilm models to accurately represent physiological conditions. Unsurprisingly, antigen discovery and validation using such systems often translate poorly into both in vivo and clinical studies. Subsequently, current vaccine solutions often attempt to prevent disease by averting the initial colonization of bacteria, and antibiotics are prescribed to treat infections caused by bacteria that have dispersed from biofilms (e.g., pneumonia and bacteremia); however, these strategies provide an opportunity for niche replacement by non-vaccine-type bacteria and increased antibiotic resistance, respectively. In this article, we provide an overview of the role that biofilms play in bacterial infections and the limitations of current models used to study them. We then highlight recent developments that have improved the accuracy of biofilm models and provide recommendations for using such models for the development of improved vaccine and therapeutic strategies.

Key Points

Human diseases caused by colonizing bacterial pathogens remain hard to treat due to the complexity of the biofilm communities they form.

We summarize how a better understanding of these communities, along with advances in biofilm models and reverse vaccinology, can be applied towards the development of innovative anti-biofilm vaccines.

1 Introduction

It is well-understood that bacteria can form complex communities known as biofilms; however, in recent years it was discovered that numerous diseases result from bacteria released by these biofilm structures as opposed to the structure itself [1]. This discovery advanced our understanding of widely prevalent conditions, such as pneumococcal pneumonia, meningitis, and otitis media, all of which have been linked with biofilm formation [2]. According to the National Institutes of Health, biofilms are associated with 80% of all infections [3]. As a result, a new

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appreciation for the involvement of biofilm formation in bacterial infections has given way to a broader field of research focused on the characterization of biofilms in physiological environments and their role in disease progression.

When combatting bacterial illnesses, an array of prescribed antibiotics have been successful at treating acute infections associated with planktonic bacteria (i.e., floating as single cells in solution). However, most antibiotics do not effectively clear bacterial biofilms due to the protection offered by the biofilm matrix, as well as the formation of metabolically dormant persister cells that can repopulate the biofilm [4]. Due to the challenges associated with using antibiotics to clear biofilms, vaccines have been developed that specifically seek to prevent biofilm formation. This is exemplified by the Prevnar family of pneumococcal conjugate vaccines (PCVs), which have significantly reduced the incidence of invasive pneumococcal disease (IPD) in countries with a high vaccination rate, such as the USA [5, 6]. In Prevnar 7 (PCV7) and Prevnar 13® (PCV13), capsular polysaccharides (CPSs) expressed during colonization are chemically conjugated to an immunogenic protein (CRM197). At the time of introduction, the selected CPSs in these vaccines were implicated in over 80% of IPDs [7]. Although this strategy dramatically reduced the rate of IPD, there has been an increase in IPDs caused by less virulent non-vaccine-type (NVT) serotypes due to niche replacement [8]. While the impact of these NVT serotypes is moderate when compared to the vaccine-type serotypes [8], this phenomenon raises concerns about the long-term efficacy of vaccine strategies based on antigens with variability across bacterial strains.

The development of a truly comprehensive vaccine could account for the numerous complexities associated with the biofilm phase, such as strain and phenotype diversity seen during the growth and release phases to provide direct protection directly against the disease-causing phenotype of bacteria. One significant challenge in the development of vaccines against biofilm-associated bacteria is the poor translation of current models with the in vivo reality [9]. Consequently, data collected using these models, such as transcriptional analysis, may be misleading and hinder antigen discovery. To overcome these challenges, this review proposes an application of improved methodologies for creating biofilms models that more accurately represent in vivo colonization and enable the application of reverse vaccinology towards the development of truly selective vaccines against biofilm-forming pathogens.

2 Selecting Which Stage of Pathogenesis to Target

One of the challenges to developing selective vaccines against biofilm-forming bacteria is identifying which stage of pathogenesis should be targeted. Targeting specific stages of pathogenesis may provide different advantages to combating disease and may differ across bacterial species. However, the biofilm formation process can be generalized for all bacterial species into four stages: (1) reversible adhesion; (2) irreversible attachment; (3) maturation; and (4) dispersion (Fig. 1) [10]. These stages can be considered individually when designing targeted vaccines.

As a biofilm develops, each phase will exhibit unique characteristics that must be considered when developing vaccines against biofilm-forming bacteria. The initial reversible adhesion of colonizing bacteria, for example, is non-specific to host cell type [11] and is typically promoted by environmental factors such as temperature, pH, and charge interactions between the pathogen and host cell [12]. Afterwards, a fraction of loosely adherent cells will become irreversibly attached with the aid of various bacterial factors, such as surface anchor proteins [13] and pili [14–16]. Following the initial adhesion to host tissue, the biofilm structure matures as the cells within the community produce a matrix composed of polysaccharides, extracellular DNA (eDNA), lipids, and proteins that are collectively termed the 'extracellular polymeric substances' (EPS) [17]. After maturation of the biofilm, bacterial cells can disperse from the biofilm matrix in response to environmental cues that include changes in temperature, pH, nutrient concentration, biofilm cell density, and host tissue damage [18–22]. This release of bacteria leads to the development of invasive diseases (e.g., sepsis) as the bacteria disseminate to normally sterile anatomical regions, causing episodes of acute infections (Fig. 1) [23].

Identifying the optimal phase of biofilm development that should be targeted in vaccine and therapeutic development is also highly dependent on the clinical manifestation of the bacteria. For example, *Staphylococcus aureus* infections include soft tissue and skin infections, pneumonia, osteomyelitis, endovascular infections, septic arthritis, surgical implant-/foreign body-associated infections, septicemia, and toxic shock syndrome [24]. In treating surgical implant infections, it is preferable to target either the adhesion or mature biofilm phase [25]. However, the other infections listed, including soft tissue and skin infections, have been linked with bacterial dissemination from asymptomatic *S. aureus* biofilms located within the nasal cavities [26, 27]. Consequently, a vaccine targeting nasal *S. aureus* biofilms would provide an effective approach to reducing the burden of diverse staphylococcal

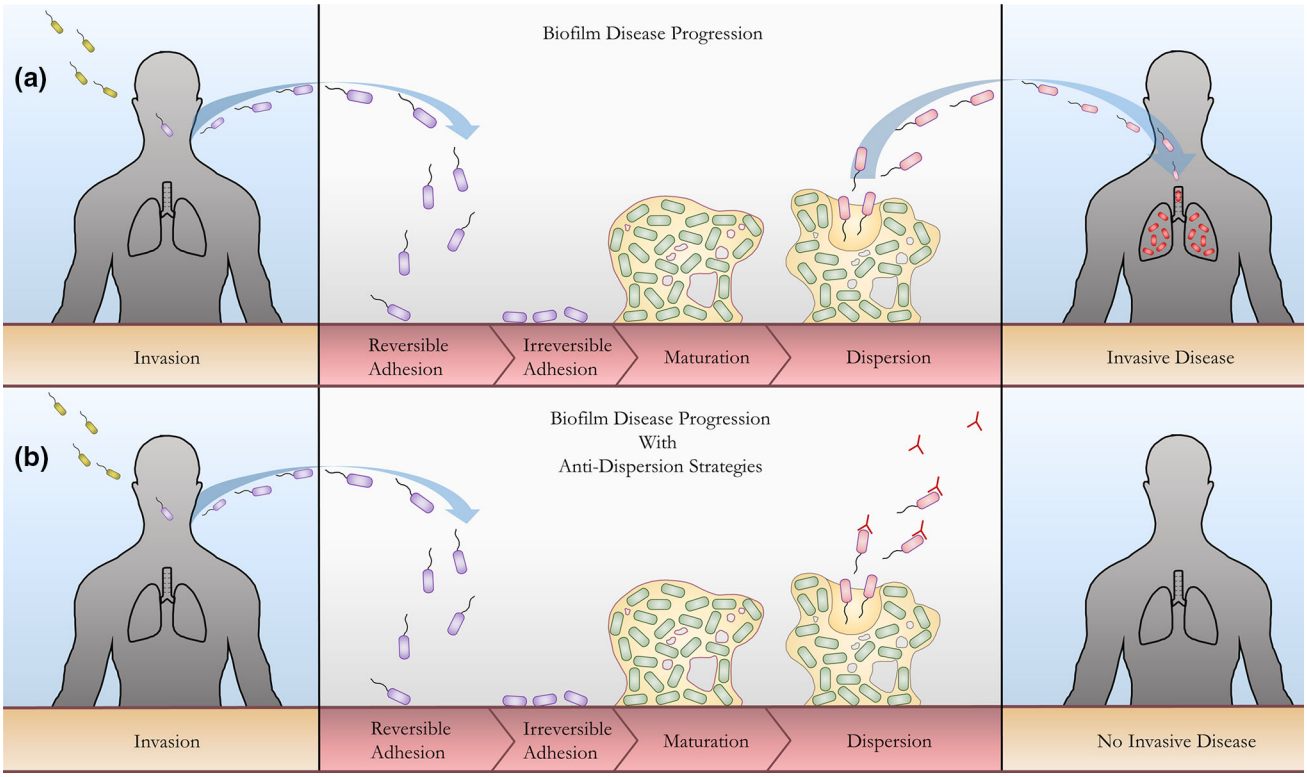

Fig. 1 Overview of the pathogenesis of biofilm-producing bacteria (**a**) and targeted interventions that preserve asymptomatic colonization while targeting the virulent dispersed phenotype (**b**)

diseases since these colonies represent a common initial stage among the various infectious caused by *S. aureus*.

The association between staphylococcal infections and the release of *S. aureus* from biofilms provides further confirmation of studies conducted on *Streptococcus pneumoniae* that demonstrated the importance of biofilms in progression of bacterial diseases [21, 28]. For example, one study showed that influenza A virus (IAV) infections or elevated temperatures (i.e., febrile) induced dispersion of virulent *S. pneumoniae* which led to bacterial pneumonia [21], a behavior that was later observed in *S. aureus* [29]. Despite the accepted belief that biofilm dispersion is critical to the spread of infectious disease [22, 23, 30], this phase has been overlooked in vaccine design in the past (e.g., Prevnar). Since biofilms formed by nasopharynx colonizers are often asymptomatic and could potentially offer protection against opportunistic pathogens [31], vaccine strategies specifically targeting biofilm-released bacteria have potential advantages.

3 Developing In Vivo and In Vitro Biofilm Models

To develop and evaluate novel vaccines accounting for the biofilm phase, it is essential to possess biofilm models that accurately represent physiological conditions. Currently, biofilm studies can be segmented into two primary categories: in vitro and in vivo models. For in vitro models, the most common adhesion surfaces are abiotic [32]. However, results from studies characterizing biofilms on abiotic surfaces, such as plastics, have shown negligible correlation to in vivo conditions except when modelling biofilms on medical implants. Consequently, using conventional in vitro models for the study of gut or nasopharynx biofilms is generally impractical [33, 34]. In addition, bacteria isolated from these in vitro models demonstrate reduced virulence when introduced into in vivo models [21, 35]. These cells also tend to form less structured biofilms that are more susceptible to antibiotics and reduce the trustworthiness of such studies for vaccine development [34]. These results show that any virulence or pathogenesis data obtained in such in vitro systems is unreliable in a clinical setting.

Models containing biological adherent surfaces, such as tissue cultures, provide a more accurate representation of the environments found within the host than their abiotic counterparts and represent a more realistic biofilm formation. For example, a study conducted by Marks et al. [34] demonstrated that *S. pneumoniae* biofilms grown on human nasopharynx cells showed extracellular matrix production, antibiotic resistance, and the formation of highly structured colonies similar to those observed in vivo. However, it has been shown that some bacterial species, such as

Pseudomonas aeruginosa, grown in this manner can dedifferentiate and lose specialization [36], which is detrimental if bacterial adhesion is dependent upon a specific host cell surface compound [37]. An interesting recent alternative is the development of three-dimensional models that incorporate live tissues or organs [36, 38]. One particular study utilized a biocompatible scaffold to culture bronchial epithelial cells, which was used in a bioreactor environment that was capable of recreating a non-typeable *Haemophilus influenzae* (NTHi) infection with the invasive phenotype observed in human explants [39]. While there are limitations to utilizing in vitro models to study bacterial biofilms, this last example demonstrates that methods which closely mimic the host environment yield more relevant outcomes.

In contrast to in vitro models, biofilm studies conducted in in vivo animal models provide an even more accurate representation of disease pathogenesis in a biological host. Furthermore, specific animal models have been developed and validated for their capacity to best mimic the complex interplay between host and pathogen [38, 40]. Chinchillas, for example, have become the golden standard in vivo model for otitis media since their ear structure is large, easily accessible, and is highly similar to that of humans [38, 41]. In addition, chronic lung infections have been replicated in cystic fibrosis (CF) C57Bl/6NCrl mice by immobilizing *P. aeruginosa* on agar beads and intratracheally installing these beads into the lungs. This technique mimics the biofilm formation in CF patients and can be used to evaluate potential therapeutics [42]. However, such animal models are often limited by their immunological differences from humans (e.g., mice and rats) [43]. For example, clinical isolates of biofilm-forming pathogens, including many strains of *S. pneumoniae*, are incapable of colonizing or causing invasive disease in rodents [44], making it difficult to accurately test the potential efficacy of a vaccine.

An interesting solution that overcomes the limitations of the models discussed above is the combination of in vivo and in vitro models. Klug et al. [45], for example, demonstrated that biofilm bacteria, when introduced into in vitro systems, were able to retain aspects of their natural complexity. Transplanting bacteria from in vitro surfaces into in vivo models has also proven effective at closely mimicking bacterial disease pathogenesis in humans. This has recently been demonstrated using strains of *S. pneumoniae* which cannot naturally cause infectious disease in mice. When clinical isolates of *S. pneumoniae* are conditioned using an in vitro biofilm release model and introduced into a murine model, these strains demonstrated characteristic colonization and disease progression to both sepsis and pneumonia. Consequently, this model allows for in vivo vaccine efficacy studies against clinical isolates that are normally not infectious in mice [44]. This enabled the development of a compressive opsonophagocytic assay (OPA), which is the most widely accepted correlate of protection for pneumoniae [46].

4 Rational Selection of Vaccine Antigens

One of the most important stages in vaccine development is the initial selection of the antigen(s). However, this step is often complicated by the dynamic gene expression profiles that are observed throughout biofilm development [47]. In the context of vaccine design, this means that a single antigen is unlikely to address all phases of biofilm formation and dispersion, often requiring vaccines to target a specific stage, as discussed in Sect. 2. Many recent vaccination attempts have made use of adhesion-associated antigens to prevent colonization, as demonstrated by the use of pili antigens to immunize against *Streptococcus pyogenes* (Table 1), thus reducing the risk of disease while providing herd immunity [48]. This strategy has also been exemplified by the PCVs developed by Pfizer, which utilize CPSs expressed during the adhesion phase [49, 50] and shed shortly thereafter [51]. However, as these vaccines only target up to 13 of the >95 disease-causing serotypes, NVT *S. pneumoniae* can still adhere, form biofilms, change serotypes (i.e., CPS), and cause disease [52].

Bacterial cells within maturing biofilms can be further broken down into discrete phenotypes, which can complicate development of vaccines targeting the mature biofilm. For example, it has been demonstrated that gene expression within cells in mature biofilms are dependent upon the cell's location within the biofilm [53]. This is due to the expression of potential antigens that are influenced by nutrient and oxygen gradients present within the biofilm [53, 54]; thus, any vaccine developed using such antigens would potentially ignore cells not expressing the antigen, hindering the vaccination efficacy. This was demonstrated by a study conducted by Brady et al. [55], which analyzed four antigens expressed by *S. aureus* (Table 1). The authors concluded that each protein was not homogeneously expressed throughout the biofilm [55], thus demonstrating the need to confirm universal expression of an antigen throughout the biofilm or develop multivalent vaccines to cover all phenotypes within the colony. Otherwise, vaccines will fail to completely clear the bacteria and the resulting biofilm disruption could lead to infectious disease.

Table 1 Vaccines targeting bacterial biofilms

Targeted bacteria	Vaccine	Clinical status	Mechanism	References
Staphylococcus aureus	Lipase, SA0486, SA0037, SA0688, and glucosaminidase	Preclinical	Targets mature biofilms	[55]
Streptococcus pneumoniae	Prevnar 13®	Approved	Prevents colonization by targeting capsular polysaccharides	[49]
	Pneumovax® 23	Approved	Prevents colonization by targeting capsular polysaccharides	[50]
	GlpO and PncO	Preclinical	Targets virulent, biofilm-dispersed bacteria	[44]
Neisseria meningitidis	Bexsero®	Approved	Immunization with NadA and NHBA prevents bacterial adhesion	[68, 69]
Streptococcus pyogenes	Pilus-forming proteins of M1_SF370	Preclinical	Prevents bacterial adhesion	[48]

NadA neisserial adhesin A, *NHBA* neisserial heparin-binding antigen

5 Application of Reverse Vaccinology

One method with the potential to overcome the antigen selection challenges introduced by genetic diversity in the biofilm phase is a process known as reverse vaccinology. This strategy involves the use of proteomics and genomics to analyze bacterial protein expression and predict optimal vaccine antigens [56, 57]. Selection criteria, such as surface accessibility, non-homology to human proteins, B cell and T cell epitopes, and homology across bacterial strains, are often applied during reverse vaccinology [58, 59]. Each of these criteria have a large impact on the efficacy of a vaccine. Surface accessibility of an antigen, for example, allows for antibodies generated by immunization to interact with surface-exposed proteins on a pathogen [60]. An ideal vaccine antigen also must not have homology to human proteins, thus preventing the generation of autoimmunity, and requires epitopes for B and T cells that elicit a strong immune response [61]. Finally, to cover as many strains as possible, a vaccine antigen should be conserved amongst strains, which is a good indication of broad coverage [62].

The first pathogen subjected to reverse vaccinology was *Neisseria meningitidis* (Table 1), a common cause of bacterial meningitis. Utilizing whole-genome sequencing, 600 potential antigens were identified and tested for immunogenicity [63]. Through this high-throughput characterization, five antigens were selected and combined into the 5CVMB vaccine [64]. This vaccine was later combined with an outer membrane vehicle to create the 4CMenB vaccine [65, 66], which is now licensed under the name Bexsero® [67–69]. More recently, reverse vaccinology was employed to characterize potent and selective vaccine antigens for *S. pneumoniae*. Using transcriptomic data, researchers identified antigens that were upregulated during both biofilm and biofilm-released phases [20]. From the transcriptomic data, Li et al. [44] identified two surface-accessible antigens that were upregulated during biofilm release; thus, the resulting vaccine was able to specifically target only the virulent biofilm-released bacteria while leaving the asymptomatic biofilm in place (Table 1). Both proteins were conserved throughout pneumococcal strains and that elicited universal protection against pneumococcal pneumonia in murine models [44].

6 Conclusion

The considerations enumerated in this article will enable researchers to specifically target any phase of disease pathogenesis associated with biofilm-forming bacteria. This could potentially usher in a new generation of smart vaccines that effectively combat pathogens that have evaded prior vaccination efforts. The development of an *S. aureus* vaccine, for example, has been complicated by the vast number of virulence factors associated with *S. aureus* and the diversity of diseases it causes [54]. However, studying how these bacteria colonize and disseminate from biofilms located in their major reservoir, the nasal passages [24], and identifying potential antigens associated with each phase can facilitate the development of vaccines targeting specific or diverse staphylococcal infections. However, there are still many aspects of biofilm formation that are poorly understood, such as gene expression within multispecies biofilms and host–pathogen interactions, which hinder vaccine development. Overcoming these limitations while taking the complexity of physiological biofilms into consideration has the potential to dramatically improve the development of vaccines and therapeutics against bacterial pathogens.

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Compliance with Ethical Standards

Conflict of interest C.H.J., A.H., and B.A.P. are co-founders of Abcombi Biosciences Inc., a company focused on vaccine design. M.B. and P.R. declare that they have no competing interests.

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