Vaccine development in *Staphylococcus aureus*: taking the biofilm phenotype into consideration

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Abstract

Vaccine development against pathogenic bacteria is an imperative initiative as bacteria are gaining resistance to current antimicrobial therapies and few novel antibiotics are being developed. Candidate antigens for vaccine development can be identified by a multitude of high-throughput technologies that were accelerated by access to complete genomes. While considerable success has been achieved in vaccine development against bacterial pathogens, many species with multiple virulence factors and modes of infection have provided reasonable challenges in identifying protective antigens. In particular, vaccine candidates should be evaluated in the context of the complex disease properties, whether planktonic (e.g. sepsis and pneumonia) and/or biofilm associated (e.g. indwelling medical device infections). Because of the phenotypic differences between these modes of growth, those vaccine candidates chosen only for their efficacy in one disease state may fail against other infections. This review will summarize the history and types of bacterial vaccines and adjuvants as well as present an overview of modern antigen discovery and complications brought about by polymicrobial infections. Finally, we will also use one of the better studied microbial species that uses differential, multifactorial protein profiles to mediate an array of diseases, *Staphylococcus aureus*, to outline some of the more recently identified problematic issues in vaccine development in this biofilm-forming species.

A history of bacterial vaccines

The first bacterial vaccines developed used whole bacteria in either a live, attenuated vaccine (LAV) or a killed, whole-cell vaccine (KWCV). LAVs are generated either by repeat passage of the pathogen in a nonstandard host or in culture media or more recently by the targeted deletion of gene(s) that enable a pathogenic phenotype in humans. Louis Pasteur’s work on the chicken cholera bacterium (*Pasteurella multocida*) and anthrax are the earliest examples of bacterial LAVs. Subsequent research on bacterial LAVs led to the development of the BCG vaccine for tuberculosis (Bastos *et al.*, 2009), the salmonella Ty21a vaccine for the prevention of typhoid (*Wahdan et al.*, 1980), and the CVD103-Hgr vaccine against cholera (*Ketley et al.*, 1993; Levine & Kaper, 1993). These vaccines continue to be used in developed and developing countries, because LAVs often confer a robust, long-lasting protection without the need to administer frequent booster shots.

Salmon and Smith subsequently laid the foundation for administering a heat-killed suspension of bacteria and paved the way for KWCVs. These vaccines were easy to produce, but had frequent adverse effects such as fever, anorexia, and swelling or induration induced by lipopolysaccharide. These drawbacks have led to almost complete clinical disuse of KWCVs in the United States. In response to these side effects, acellular, protein versions of traditional vaccines such as the acellular pertussis vaccines (Decker & Edwards, 2000) and the acellular anthrax vaccines (Friedlander & Little, 2009) followed. Rationales for immunizing with a limited number of antigens are reduced reactogenicity and avoidance of autoimmunity resulting from molecular
mimicry by bacterial antigens (Zorzeto et al., 2009). A limitation is that immunity elicited by a single antigen wanes more quickly than that generated by a LAV.

Alternatively, the tetanus and diphtheria toxoid vaccines developed in the 1920s are currently being used with minor alterations to their manufacture (Plotkin et al., 2008). The toxoid vaccine lacks the toxin's pathogenic qualities and is used for vaccination to generate neutralizing antibodies against the toxin. Because single toxins are responsible for the bulk of Clostridium tetani and Corynebacterium diphtheriae pathogenesis, a robust immunoglobulin G (IgG) neutralizing antibody response that targets and blocks the toxin interrupts the disease process.

A better understanding of the critical role of polysaccharide capsules in the pathogenesis of Streptococcus pneumoniae and Haemophilus influenzae led to the development of polysaccharide vaccines (PSVs) against these pathogens (Riley et al., 1977; Robbins et al., 1983; Mufson et al., 1985) as well as a PSV against Neisseria meningitidis serotypes A, C, W-135, and Y (Artenstein et al., 1970; Armand et al., 1982; Ambrosch et al., 1983). Because of suboptimal immunogenicity elicited by polysaccharide, PSVs are being eliminated and replaced by polysaccharide–protein conjugate vaccines. Conjugate vaccines elicit a robust IgG response imparted by the protein carrier, which converts the polysaccharide from a T-cell-independent immunogen into a T-cell-dependent immunogen (Perez-Melgosa et al., 2001).

Innovations to vaccine design over the years have resulted in a number of successful bacterial vaccines that supplant earlier, less effective vaccines. Currently, several competing cholera (Lopez et al., 2008) and typhoid vaccines (Fraser et al., 2007) are available. A closer examination of these vaccines defines the pros and cons of certain vaccine strategies (Table 1).

Although vaccinology has made significant progress (Table 2), many challenges remain to date. When dealing with bacterial pathogens that can cause multiple forms of diseases through a large number of virulence factors, often traded between individual strains and species by horizontal gene transfer, protection via a single component vaccine is likely to be elusive. Staphylococcus aureus is an example of such a pathogen. This microbial species has dozens of known toxins, multiple immunovoidance, and adherence factors, most of which demonstrate transient, timed, and disease-specific expression (DeLeo et al., 2009). Therefore, a successful vaccine will likely be required to provide protective antibody titers against multiple antigens (Zecconi et al., 2005).

### Types and modes of delivery of vaccines

Recombinant subunit protein technology has become the main strategy in the development of vaccines against infectious diseases. Subunit vaccines offer several advantages over previous vaccine strategies. Recombinant subunit vaccines are safe or less reactogenic with a defined composition, which is due to its genetic-based approach and antigen expression in nonpathogenic bacterial strains. Other advantages include multiple modes of delivery and further engineering of the subunit (Liljeqvist & Stahl, 1999; Hansson

| Vaccine type                  | Pros                                                                 | Cons                                                                 |
|------------------------------|---------------------------------------------------------------------|----------------------------------------------------------------------|
| Killed, whole bacteria       | Relatively simple to make, produces a protective immune response for many organisms | Highly reactogenic in many cases, has rendered vaccines unusable or unpopular, risk of induction of autoimmunity via molecular mimicry, booster doses often needed |
| Live, attenuated bacteria    | More robust and longer lasting immunity relative to killed, whole bacteria | Possibility of disease in immunocompromised patients, possibility of reacquisition of lost virulence resulting in disease |
| Toxoid                       | Excellent at generating toxin neutralizing antibodies                | Multiple doses often needed, epitope must be highly conserved         |
| Protein only                 | Markedly less reactogenic compared with killed, whole bacteria       | Multiple doses often needed, epitope must be highly conserved         |
| Polysaccharide only          | Markedly less reactogenic compared with killed, whole bacteria       | Multiple doses often needed, epitope must be highly conserved         |
| Polysaccharide–protein       | Improved antibody titers relative to polysaccharide only             | Meningococcal conjugate vaccine not currently recommended for children under age 11 |
| conjugate                    | Decreased carriage for meningococcal and pneumococcal vaccines      |                                                                      |
|                              | Can generate longer lasting immunity relative to polysaccharide vaccines |                                                                      |
|                              | Markedly less reactogenic compared with killed, whole bacteria       |                                                                      |

Table 1. General characteristics of classical bacterial vaccine types
Table 2. Common bacterial vaccines

| Pathogen (disease)          | Vaccine type               | Composition                                                                 | Current status                                                                 |
|----------------------------|----------------------------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| *Bacillus anthracis* (anthrax) | Live, attenuated           | Sterne live-attenuated strains                                              | Not available in the United States for humans, only for veterinary use          |
|                            |                            |                                                                             |                                                                                  |
|                            | Acellular                  | Cell-free culture supernatant adsorbed to aluminum hydroxide; believed to contain mostly the protective antigen of the anthrax toxins |                                                                                  |
| *Bordetella pertussis* (pertussis) | Killed, whole cell        | Killed pathogenic bacteria                                                  | Completely replaced by acellular vaccine in the United States and many developed countries |
|                            |                            |                                                                             |                                                                                  |
|                            | Acellular                  | Inactivated pertussis toxin plus one or more of the following proteins: hemaglutinin, pertactin, or fimbriae types 2 and 3 | Approved for clinical use in the United States                                  |
| *Borrelia burgdorferi* (Lyme disease) | Killed, whole cell     | Inactivated whole-cell vaccine with proprietary polymer adjuvant or bivalent whole-cell killed | Veterinary vaccines for dogs                                                    |
| *Clostridium tetani* (tetanus)               | Toxoid                     | Formaldehyde detoxified tetanus toxin                                        | Currently licensed in the United States in several combinations                |
| *Corynebacterium diphtheriae* (diphtheria)           | Toxoid                     | Diphtheria toxoid adsorbed to aluminum salt                                | Currently licensed in the United States in several combinations                |
| *Coxiella burnetii* (Q fever)                  | Killed, whole cell         | Killed *C. burnetii*                                                        | Not commercially available in the United States                                |
| *Haemophilus influenzae* type B (pneumonia and meningitis) | Polysaccharide–protein conjugate | PRP or HbOC linked to either diphtheria toxoid or the outer membrane protein complex of *N. meningitidis* | Four currently licensed conjugate vaccines in the United States                 |
|                                           |                            |                                                                             |                                                                                  |
| *Mycobacterium tuberculosis* (tuberculosis)       | Live, attenuated           | Bacille Calmette-Geurin (BCG)                                               | Widespread global use; rarely administered in the United States                |
| *Neisseria meningitidis* (meningitis)            | Polysaccharide–protein conjugate | Quadrivalent vs. A, C, Y, and W-135 strains                                | Currently licensed in the United States                                        |
| *Rickettsia rickettsii* (typhus)                | Killed, whole cell         | Inactivated chick embryo cultured *R. rickettsii*                          | No currently licensed vaccine in the United States                              |
|                                           |                            |                                                                             |                                                                                  |
| *Salmonella typhi* (Typhoid)                    | Killed, whole cell         | Heat- and phenol-inactivated *S. typhi*                                     | No longer available in the United States                                       |
|                                           | Killed, whole cell         | Acetone inactivated parenteral vaccine                                      | Only available to the United States Armed Forces                                |
|                                           | Live, attenuated           | Ty21a galactose nonfermenting *S. typhi*                                    | Available in the United States                                                 |
|                                           | Polysaccharide              | Vi capsular antigen                                                         | Available in the United States                                                 |
|                                           | Polysaccharide–protein conjugate (Vi-rEPA) | Vi capsular antigen conjugated to *Pseudomonas aeruginosa* recombinant exotoxin A | In development                                                                |
| *Streptococcus pneumoniae* (pneumonia and meningitis) | Killed, whole cell         | Monovalent killed                                                          | Abandoned, not available                                                       |
|                                           | Polysaccharide              | 6-, 14-, and 23-valent polysaccharide vaccines                               | No longer used in the United States because it couldn’t be used for children < 2 years old and superior protection was afforded by conjugate vaccines |
|                                           | Polysaccharide–protein conjugate | 7-valent polysaccharide conjugated to diphtheria CRM197 carrier protein          | Currently licensed for prevention of infant and child meningitis               |
|                                           |                            |                                                                             |                                                                                  |
| *Vibrio cholerae* (Cholera)                    | Killed, whole cell         | Killed pathogenic bacteria                                                  | Licensed for the prevention of pneumonia in patients of 65 years of age or older or immunosuppressed patients over the age of two |
|                                           |                            |                                                                             | Licensed, but not widely used                                                  |
et al., 2000). The main drawbacks of subunit vaccines are the requirement of an adjuvant and multiple doses as well as low immunogenicity and a short half-life, which can be improved by conjugating the protein subunit to another protein or molecule (Hudecz, 2001; Tugyi et al., 2008). Conjugation of an antibody, adhesion factor, or other molecule (such as cholera toxin B subunit) to the peptide can target it to immunologically relevant sites or cells to improve response. Recombinant subunit vaccine efficacy is also reliant on the route of administration.

Current delivery methods include parenteral (e.g. transcutaneous and intramuscular) and mucosal (e.g. intranasal and oral) vaccines. The skin serves as a functional barrier by preventing harmful molecules and organisms from invading the host. Langerhans cells, a class of antigen-presenting cells, present antigens in the epidermal layer and the accessibility of the skin makes parenteral vaccination a favorable delivery method. The parenteral route of vaccine delivery is an effective inducer of systemic immunity represented by significant serum IgG titers and cytokine expression in lymph nodes. Nevertheless, this mode of vaccine delivery is deficient in its ability to initiate a mucosal immune response.

The mucosal surface is resident to the majority of lymphocytes found in the human body and is also the main entry point for infectious agents. This makes targeting vaccines to the mucosal sites crucial for immunity. The main advantage of mucosal vaccination over parenteral is the induction of IgA secretion at mucosal sites in combination with systemic IgG titers. Secreted IgA prevents the colonization and invasion of pathogens and neutralizes toxins at the mucosa (Slutter et al., 2008). Mucosal vaccination leads to antigen-specific B cell memory, with the caveat that a proper immunostimulating compound is used (Vajdy, 2006). Antigen delivered without an adjuvant leads to mucosal tolerance, resulting in clonal deletion or induction of anergy of antigen-specific lymphocytes (Ogra et al., 2001). In addition to mucosal tolerance, inefficient uptake of antigen and delivery to antigen-presenting cells is another disadvantage of mucosal vaccination (Slutter et al., 2008). Mucosal vaccination has the potential to alleviate the innumerable diseases caused by pathogenic bacteria, viruses, and parasites by providing complete protection through IgA-mediated mucosal and IgG-mediated systemic immunity. Overcoming the hurdles of mucosal tolerance and inefficient antigen delivery may augment the vaccines currently in clinical trials.

### Adjuvants

Adjuvants work by stimulating the innate immune response, which is a required step in activating adaptive immunity. Cytokines and chemokines expressed upon stimulation of the innate immune response attract leukocytes to the local environment and cause maturation of antigen-presenting cells such as dendritic cells (DCs). The resident DCs are effective messengers between the innate and the adaptive response due to their enhanced antigen-presenting capabilities and ability to become polarized. Adjuvants promote cytokine expression within a microenvironment that polarizes DCs to mediate the expression of Th1 or Th2 cytokines and costimulatory molecules. In the draining lymph nodes, polarized DCs present the antigen to naive T-cells. The development of Th0 to Th1, Th2, or other T-helper cells during antigen presentation is dependent on the expression of polarizing cytokines and costimulatory receptors produced by DCs. T-cells activated during this process potentiate the subsequent adaptive immune response.

Selecting the appropriate adjuvants for vaccine development is crucial, because they play a critical role in the development and polarization of the adaptive immune response. Adjuvants have been found to favor either a Th1 or a Th2 response, suggesting the production of Th1- and Th2-polarizing cytokines at the site of administration. To
understand the immune response initiated by an adjuvant, whether it be Th1 or Th2, becomes essential in the selection of an adjuvant for vaccine design. Few adjuvants exist in the clinical realm; however, many are being tested experimentally. Table 3 details supplemental information on the current and experimental adjuvants.

Adjuvants are potent inducers of innate immunity. They are often needed for an effective and protective adaptive immune response against pathogens. The Th response stimulated by vaccination is dependent on the cytokine milieu produced locally by an adjuvant, and the resultant polarization of antigen-presenting cells. Also, planktonic vs. biofilm-mediated diseases initiated by the same pathogen complicate vaccine development as each phenotype may require different Th responses to provide postvaccination protection. Research on the immunostimulating properties of molecules will elucidate future adjuvants and provide even greater options for vaccine development.

**Novel strategies for antigen selection: highlighting *S. aureus* advances**

Vaccine design changed dramatically with advancements in genome sequencing technologies that enable rapid completion of genomes. Since the publication of the *H. influenzae* genome in 1995, the NCBI genome project reports that 1026 complete microbial genomes have been published including ones for 15 *S. aureus* strains (Fleischmann et al., 1995) (http://www.ncbi.nlm.nih.gov/genomeprj). Access to complete genomes and bioinformatic technologies to manage and analyze the data has advanced high-throughput molecular techniques for genomic, transcriptomic, and proteomic analyses of microbial growth and pathogenesis (Kaushik & Sehgal, 2008; Zagursky & Anderson, 2008). Genome-based technologies provide rapid identification of vaccine candidates compared with the conventional vaccine approaches, which identify and analyze individual virulence factors from pathogens grown in *vitro* (Rappuoli, 2000). Vaccines developed via genome-based technologies will still slowly transition into clinical phases after rapid identification, because these vaccines require the same rigorous evaluations using *in vitro* assays and animal models to validate functional activity as conventionally derived vaccines. As this review focuses on vaccine development against *S. aureus* to highlight *in vivo* phenotypes (e.g. biofilm formation and polymicrobial infection) that should be considered during antigen identification, we choose to present genome-based strategies and other technologies that identified putative *S. aureus* virulence factors and/or vaccine candidates. Vaccines comprised of antigenic candidates identified by these strategies may provide protection against *S. aureus* infection, but the overall lack of an effective *S. aureus* vaccine to date indicates that critical phenotypes and factors are not adequately addressed in current vaccines. For the strategies outlined below, both these and future studies examining alternate parameters will

**Table 3. Adjuvant-dependent effector T cell differentiation**

| Adjuvants | Clinical status | Immune response | Experimental observations to designate immune response | References |
|-----------|----------------|-----------------|------------------------------------------------------|------------|
| Alum      | Only one approved for US vaccines | TH2             | TH1: No IgG2a titer, No IFN-γ | Uddowlia et al. (2007), Brever (2006) |
|           |                 | TH2             | TH2: High IgG1 titer, IL-4 and IL-5 produced | Uddowlia et al. (2007), Brever (2006) |
| MF59      | Flud influenza vaccine* | TH2             | TH1: Low IgG2a titer | Valensi et al. (1994), Wack et al. (2008) |
|           |                 | TH2             | TH2: High IgG1, IL-5, IL-4, and THF-α produced | Valensi et al. (1994), Wack et al. (2008) |
| MF59 with CpG | No clinical application¹ | TH1             | TH1: High IgG2a titer, IFN-γ produced | Wack et al. (2008) |
|           |                 | TH2             | TH2: Low IgG1 titer, IL-5 suppressed | Wack et al. (2008) |
| AS04      | Cervarix* (HPV)–Fendrix* (Hepatitis B) | TH1             | TH1: High IgG2a, IL-2 and IFN-γ produced | Korsholm et al. (2010), Didierlaurent et al. (2009) |
|           |                 | TH2             | TH2: Low IgG1, IL-6 and THF-α produced | Korsholm et al. (2010), Didierlaurent et al. (2009) |
| c-di-GMP  | No clinical application¹ | TH1/TH2        | TH1: High IgG2a and IgG2b IFN-γ, THF-α, IL-12, MCP-1, and RANTES produced | Karaolis et al. (2007), Hu et al. (2009) |
|           |                 |                 | TH2: High IgG1 and IgG3 | Hu et al. (2009) |

*European-approved vaccine application only.
¹Not approved for human vaccine applications.
be invaluable resources to refine the search for vaccine candidates.

**Genomics/transcriptomics**

Identification of vaccine candidates through the systematic search of the genome and identification of putative antigens, mainly surface-associated proteins, using bioinformatics is referred to as 'reverse vaccinology' (Rappuoli, 2000). The progression of this field and its significance to vaccine development against serogroup B *N. meningitidis* and group B *Streptococcus* are detailed in reviews by Serruto & Rappuoli (2006), Serruto et al. (2009). This method has a number of advantages compared with previously used methods in that there is no need to grow the pathogen *in vitro* and antigen selection can proceed independent of the abundance of *in vivo* expression and immunogenicity. As a result, many unique antigens can be tested that would have been passed over in conventional studies.

Vaccine candidates identified from a single genome in reverse vaccinology must provide *in vivo* protection against multiple clinical strains in correlative animal models to support transition into clinical studies. An approach, known as comparative genomic hybridization (CGH), uses a DNA microarray of a sequenced 'reference' strain to screen for the presence or absence of genes within nonsequenced 'test' strains and limits the candidates to antigens conserved in multiple strains. However, the modern ability of advanced sequencing methods such as pyrosequencing has enabled whole-genome sequencing for multiple genomes from various strains of a microbial species to become commonplace. Access to complete genomes of multiple strains for some bacteria makes sequence comparisons among multiple genomes a favorable alternative to CGH because the comparison accounts for all genes within each strain. Earlier CGH studies and more recent deep strain sequencing have led to a description of the 'pangenome' in three parts: a 'core' genome comprised of genes conserved in all genes, a distributed genome composed of genes not conserved in one or more strains, and a subgroup comprised of novel genes encoded by a single strain (Tettelin et al., 2002, 2005; Shen et al., 2005; Ehrlich et al., 2008). A protective quadrivalent vaccine for *S. aureus* was assembled from surface proteins, IsdA, IsdB, SdrD, and SdrE, after searching eight genomes and evaluating the protective efficacy of multiple candidate antigens in mice (Stranger-Jones et al., 2006).

The increased access to complete genomes of bacteria has led to the ability to develop unique cDNA microarrays for transcriptomic profiling. Evaluation of the bacterial transcriptome under *in vitro* conditions, mimicking environmental stimuli encountered during host infection, detects upregulated genes that may represent virulence factors and vaccine candidates. Transcriptomic analysis is generally restricted to *in vitro* studies, because bacterial RNA is difficult to extract differentially from the infected host tissue.

**Gene expression technologies: positive selection**

Other technologies make use of the *in vivo* transcriptional profiles to gather information on the genes involved in virulence, but circumvent the restrictions of RNA extraction and microarray analysis. Three techniques that analyze *in vivo* gene expression and predict promising vaccine candidates are *in vivo* expression technology (IVET), differential fluorescence induction (DFI), and *in vivo* induced antigen technology (IVIAT) (Mahan et al., 1993; Valdivia & Falkow, 1996; Handfield et al., 2000).

The first report of IVET applied to a Gram-positive species was a study of *S. aureus* by Lowe et al. (1998), using a variation known as recombination-based IVET (RIVET). In the RIVET system, random genomic fragments are fused to a promoterless resolase gene, such as *tnpR*, to construct a genomic library, and a gene cassette comprised of an antibiotic resistance gene flanked by resolase recognition sequences is incorporated into the bacterial genome. Excision of the antibiotic marker from the bacterial genome, or 'resolution', is dependent on the expression of the *ivi* gene-resolase fusion, and confers antibiotic sensitivity to the bacterium (Angelichio & Camilli, 2002). Lowe et al. (1998) assessed 11 mutants for *ivi* genes that were identified from *S. aureus* genomic libraries screened in a murine renal abscess model and defined seven mutants with attenuated virulence compared with wild-type *S. aureus*. DFI is another promoter-trap approach where promoter induction controls the expression of green fluorescent protein, and microorganisms with gene expression can be isolated by fluorescence-activated cell sorting (Valdivia & Falkow, 1996). Finally, the IVIAT system screens *in vitro* expression libraries of a pathogen with convalescent sera following depletion of antibodies specific to that pathogen grown under *in vitro* conditions.

**Gene expression technologies: negative selection**

Signature-tagged mutagenesis (STM) identifies the genes required for *in vivo* growth and survival by screening heterogeneous pools of mutants. Each of the mutants has a transposon with a unique oligonucleotide tag randomly incorporated into their genome. After inoculating pools of mutants into a relevant *in vivo* infection model, those mutants that fail to colonize the model can be identified by their unique transposon tag (Hensel et al., 1995). STM screens of *S. aureus* virulence in murine models of bacteremia, abscess, and wound and rabbit endocarditis have been
completed, and report that < 10% of the mutants were attenuated in all three murine models (Mei et al., 1997; Coulter et al., 1998).

**Proteomics**

Proteomic profiling examines and identifies the spectrum of proteins expressed in bacteria under varying growth conditions using two-dimensional gel electrophoresis (2DGE) and MS. Detection of membrane and cell wall proteins is a limitation of proteomic profiling due to low abundance and solubility constraints that are caused by protein hydrophobicity, transmembrane domains, and an alkaline isoelectric point (Fountoulakis & Takacs, 2001). Because vaccine strategies focus on surface-associated proteins, proteomic analyses yield limited vaccine candidates unless extraction protocols that solubilize membrane proteins or isolectric focusing performed in the alkaline pH range are used. Reference maps of *S. aureus* Phillips and VISA surface proteomes following lysostaphin extraction have been published, and among these, membrane- and cell wall-associated proteins are promising candidate antigens that can be tested for immunogenicity and/or protective activity (Nandakumar et al., 2005; Gatlin et al., 2006). Another strategy, considered a 'new chapter in reverse vaccinology', developed concurrently with the cited work of Nandakumar and colleagues, and Gatlin and colleagues examined surface proteins 'shaved' from group A Streptococcus using trypsin digestion (Musser, 2006; Rodriguez-Ortega et al., 2006). Cell surface shaving proteomics has recently established 42 *S. aureus* COL surface proteins that may have potential for vaccine development (Solis et al., 2010).

Serological probing of proteomic samples, known as immunoproteomics, followed by peptide identification using matrix-assisted laser desorption ionization time-of-flight MS is a direct method for defining antigenic proteins. An initial 2DGE immunoproteomic study of *S. aureus* COL identified 15 known and novel proteins that were immunoreactive with patient sera (Vtvytyska et al., 2002). Using subtractive proteome analysis, Glowalla and colleagues selected proteins that were immunoreactive with an intravenous immunoglobulin (IVG) preparation and nonreactive with IVG depleted of *S. aureus*-specific opsonizing antibodies and identified three anchorless cell wall proteins that provided partial protection in a mouse sepsis model (Glowalla et al., 2009). These anchorless wall proteins lack a conserved signal peptide or an LPXTG motif, characteristic of most surface-associated proteins, and in some cases, may be consequently omitted from classical reverse vaccinology screens (e.g. vaccine development from genome analysis) (Chhatwal, 2002). Immunoproteomic studies have also evaluated two obstacles to the clinical control and prevention of *S. aureus*, biofilms that potentiate chronic infections and colonization or human carriage (Brady et al., 2006; Holtfreret et al., 2009). Indeed, most humans possess pre-existing circulating antibodies against major *S. aureus* virulence factors that do not protect against a subsequent challenge by this pathogen. Incomplete protection may be attributed to the transient nature of virulence factor expression during the infection, which requires consideration during the process of vaccine development.

**Antigenomics**

Antigenomic screens probe *Escherichia coli* surface-expressed fusions that express randomly fragmented genomic libraries with human sera that are depleted of *E. coli*-specific antibodies. The screens identify a large repertoire of antigenic peptides including those encoded by alternate reading frames (Etz et al., 2002). Indeed, antigenomic studies of *Staphylococcus* and *Streptococcus* found that 24% of antigens were hypothetical proteins or proteins of unknown function from nonannotated reading frames (e.g. alternative reading frame, complementary strand reading frame, nongene matching reading frame), which are categories eliminated from bioinformatics-based vaccine development (Meinke et al., 2005). Antigenomic peptides can be evaluated for widespread in vivo expression, or reactivity, via screening with multiple serum samples and conserved expression among multiple bacterial strains (Etz et al., 2002). High-throughput screening methods that circumvent the restrictive in-frame cloning step and peptide insolubility issues that limit peptide repertoire in the bacterial surface expression systems include phage display and ribosome display. However, antigenomic strategies may inadequately define antigenic peptides compared with in vitro expression systems, possibly due to protein toxicity and reduced membrane permeation obstructing surface expression and limiting antigen detection.

**Taking into account the mode of growth: biofilm vs. planktonic**

The early pioneering work and the continued modern era of biofilm disease discovery by a number of investigators have transformed the field of medical microbiology (Nickel et al., 1985a, b, 1986a, b, 1989; Post et al., 1996; Ehrlich et al., 2002; Erdos et al., 2003; Murphy et al., 2005; Stoodley et al., 2005; Hall-Stoodley et al., 2006; Hiller et al., 2005; Hogg et al., 2007). Because of these studies, the biofilm mode of growth has been recognized as the major mode of infection, with an estimated 80% of all infections caused by biofilms (National Institutes of Health, 1998, 1999). Although extensive studies have been performed on biofilm infections, the resolution of these infection continues to be the surgical removal of the nidus of infection (Shirliiff & Mader, 2000). This surgical removal is necessary because these microbial
communities are 50–500 times more resistant to antimicrobial agents than their planktonic and free-floating counterparts (Nickel et al., 1985b; Stewart & Costerton, 2001). Although the significance of biofilm infections has been recognized as an important mediator of chronic infection and the resulting morbidity and mortality, vaccine studies have often ignored biofilms in discovery and efficacy studies.

For example, recent vaccine development programs for S. aureus have tended to focus on testing the ability of target antigens to protect the host from in vitro or murine planktonic infection models (Fattom et al., 1996, 2004; McKenney et al., 1998, 1999, 2000; Stranger-Jones et al., 2006; Bubeck Wardenburg & Schneewind, 2008; Lin et al., 2009; Kim et al., 2010). Infections with S. aureus may exist in a biofilm mode of growth either during nares carriage or skin infections. Once transmitted to the circulatory system through an epithelial breach, planktonic growth ensues, where upregulation of adherence factors occurs (Beenen et al., 2004). At this point, the invading staphylococci are either removed by the host innate immune response or attach to host extracellular matrix proteins and develop a localized biofilm community. Once this community develops, the proteome of the microorganisms quickly transforms into a biofilm phenotype. Therefore, the planktonic mode of growth that occurs in sepsis may be a transient state. Also, although the host may be vaccinated against planktonic antigens, they may develop a significant memory response only after the secondary foci of biofilm infection has already occurred and the antigenic nature of this pathogen has also significantly changed, thereby detracting from vaccine efficacy.

In the context of biofilm infections, the first question that must be answered when selecting antigen targets is which component of the biofilm should be targeted. Broadly speaking, two alternatives exist: bacterial cells within the biofilm and the biofilm matrix itself. The biofilm matrix may be composed of polysaccharides, protein, or extracellular DNA, in proportions that vary between bacterial genera, species, and strains. As of 2009, the majority of antibiofilm vaccine efforts have been directed toward the biofilm matrix (Schaffer & Lee, 2008). Perhaps the best example of this is the staphylococcal polysaccharide intercellular adhesin (PIA), which is composed of poly-N-acetyl-β-1,6-glucosamine (PNAG). The enzymes that catalyze the production of these polysaccharides are encoded for by the genes of the icaA/BC locus (Joyce et al., 2003). PIA is produced by both Staphylococcus epidermidis (McKenney et al., 1998) and S. aureus (Cramton et al., 1999), and is known to be involved in the adherence of S. epidermidis to both host tissues (Costa et al., 2009) and inert biomaterials (Olson et al., 2006). PIA/PNAG plays an additional role in immune evasion in both the biofilm and the planktonic mode of growth. The icaA/BC locus has been detected in clinical S. epidermidis isolates (Ziebuhr et al., 1997), and its contribution to pathogenesis has been demonstrated in animal models of infection (Rupp et al., 1999). Hence, upon a superficial review, PIA would seem to be an ideal candidate for a vaccine antigen.

In contrast to S. epidermidis, PIA production is less pronounced in most S. aureus strains and often observed in vitro only under particular conditions, such as anaerobiosis (Cramton et al., 2001) or relatively high (1%) glucose concentrations (Ammendolia et al., 1999). In one study, only 57% of strains that were icaA/BC positive by PCR analysis (Arciola et al., 2001a) produced a biofilm when cultured in vitro (Knobloch et al., 2002), suggesting distinct strain differences in any correlation of PIA and biofilm formation. In vivo, analysis of clinical S. aureus isolates from prosthetic-joint infections, bacteremia (Fowler et al., 2001), catheter-related infections (Arciola et al., 2001a), or from randomly selected clinical isolates (Martin-Lopez et al., 2002) indicates possession of the ica locus by the majority of isolates. However, a lack of PIA production was observed in many of these strains in vitro. The proportion of ica-positive strains among S. aureus clinical isolates is thought to vary according to the clinical origin of the isolate and even between infection sites that are both biofilm mediated. For example, the proportion of icaA/BC-positive S. aureus strains was higher in orthopedic prosthesis-associated infection (92%) than in catheter-associated infections (63%) (Rohde et al., 2001). Thus, the site and composition of indwelling biomaterials may act as selective factors for strains with different and alternate adhesion mechanisms. The situation is further complicated by the fact that possession by a staphylococcal strain of the icaA/BC locus does not necessarily mean that PIA will be produced in vivo. Similarly, the production of PIA in vitro does not mean that it will be produced in vivo during an infection. In addition, in vitro PIA expression may differ between assays (Rohde et al., 2001). Although there is some evidence that suggests a correlation between icaA/BC possession and slime production in vitro (Arciola et al., 2001b), more research is required to fully understand the importance of PIA in staphylococcal infection in vivo. There is also limited evidence that suggests that PIA expression can undergo phase variation (Ziebuhr et al., 1997).

A vaccine based on PIA has undergone trials in animal models. McKenney et al. (1998) used PNAG to immunize mice. Five days after an intravenous challenge with two S. aureus strains (CP5 Reynolds and CP8 MN8), both of which are negative for PNAG production in vitro, immunized mice showed a significant reduction in CFU recovered from the kidneys as compared with the controls (McKenney et al., 1999). Further work by the same group suggested that the deacetylated form of PNAG, dPNAG (15% acetylation), conjugated to the diphtheria toxoid is more effective as a

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vaccine than the 90% acetylated form (Maira-Litran et al., 2005). This is likely due to the retention of dPNAG on the bacterial cell surface, in contrast to the highly acetylated PNAG form, which is released into suspension (Cerca et al., 2007). The deacetylase activity of the icaB gene product (Vuong et al., 2004) mediates this effect. The use of PNAG as a vaccine has shown promise in subsequent studies in animal models of S. aureus mastitis (Perez et al., 2009) and S. aureus skin abscess (Gening et al., 2010). Given that PNAG is produced by a variety of other bacterial taxa, including E. coli (Wang et al., 2004), Actinobacillus actino-
mycetemcomitans, Actinobacillus pleuropneumoniae (Kaplan et al., 2004), Bordetella spp. (Parise et al., 2007), and Acinetobacter baumannii (Choi et al., 2009), PNAG has shown promise in subsequent vaccine studies in animal models of E. coli bacteremia (Cerca et al., 2007) and peritonitis (Gening et al., 2010).

The efficacy of a PNAG-based vaccine against S. aureus biofilm-type infection remains to be elucidated. However, given that possession of the icaAIDBC locus by clinically isolated S. aureus varies between infection sites (Rohde et al., 2001), PNAG may not be the ideal vaccine antigen in a formulation intended to prevent biofilm-type infections. Besides PIA/PNAG, other biofilm factors have simply not been evaluated extensively and these may potentially be inappropriate targets in subsequent studies. Also, one may question whether it would be more efficacious to promote the host immune response to attack the cells producing the matrix or attack the matrix itself. The extracellular matrix of a biofilm community exists, at least in part, to act as an immunoavoidance mechanism. Furthermore, in many cases, the matrix material is constantly being produced and sloughing off into the environment.

**Polymicrobial diseases: considerations for vaccine development**

Although many infectious diseases are initiated by a single pathogen or virulence factor, others originate from or are attributed to a complex milieu of microorganisms. Examples of diseases associated with both polymicrobial and biofilm phenotypes include periodontal disease, otitis media, rhinosinusitis, ventilator-associated pneumonia, and chronic wound infections (Brogden et al., 2005). These biofilm consortia of microorganisms typically coexist as combinations of highly structured communities of bacteria, viruses, protozoans, and fungi attached to biotic and environmental surfaces, where their architecture is facilitated by specific intermicrobial and host interactions (Bakletz, 1995; Viale & Stefani, 2006; Kuramitsu et al., 2007). Many of these interactions are mutually beneficial for both the host and the microorganism (e.g. the gastrointestinal and oral microbiota). However, microbial species popula-

The impact of the polymicrobial nature of a disease regarding colonization and infection should also be considered during vaccine development. A disease must first be classified as truly polymicrobial based on sufficient data from clinical studies and epidemiological records. Important criteria regarding the temporal shifts, composition, abundance, and consistency of microorganisms present throughout the entire course of the disease, from
colonization to fulminant infection, should be considered (Roberts, 1989; Tarsia et al., 2007). One must also distinguish contaminating microorganisms (pathogens or commensals) from those that initiate and propagate infection. If a disease is considered to be of a polymicrobial nature, a vaccine composed of a multivalent cocktail of antigenic proteins from all microorganisms involved in disease pathology may be warranted. Although seemingly trivial, these criteria are crucial to understanding the pathogenesis of and developing effective vaccines for multimicrobial diseases.

Polymicrobial infections represent a significant complexity in vaccine development. Two (or more) microorganisms may act synergistically or antagonistically to mediate disease while either in isolation is differentially virulent or benign (Carlson, 1983; Diebel et al., 1999). Even if a vaccination attempt successfully negates a necessary virulence factor for one pathogen (i.e. a toxin), virulence could be complemented in trans by another factor produced by a neighboring species in the polymicrobial community. In addition, the eradication of one species from the polymicrobial community may be insufficient at reducing overall disease, as another organism present may fill in the niche left behind. Alternately, a vaccination attempt targeting a virulence factor (i.e. an adhesin) for one pathogen may successfully target and eradicate a secondary pathogen within the polymicrobial infection.

Modulation of a microorganism’s pathogenicity by the polymicrobial community has important implications for vaccine development as studies for S. aureus suggest. A formidable nosocomial pathogen, S. aureus can be isolated as the single etiologic agent in a multitude of diseases (e.g. sepsis, lower respiratory tract infections, skin infections, and others) or among a polymicrobial community in the same disease types. Polymicrobial infections complicate approximately 27% of nosocomial Candida albicans bloodstream infections; among these, S. aureus is the third most common coinfecting microorganism (Klotz et al., 2007). As microbial biofilms on indwelling medical devices act as a potential nidus for planktonic release and onset of sepsis, observations of enhanced biofilm formation and differential matrix composition for S. aureus in coculture with C. albicans suggest that polymicrobial interactions may facilitate S. aureus colonization and disease onset (Harriott & Noverr, 2009). The synergistic action of C. albicans and S. aureus has also been implicated in the increased mortality of mice infected with S. aureus strains producing the toxic shock toxin (Carlson, 1983). Indeed, vaccination against C. albicans using the candidal adhesion Als3P can provide cross-kingdom protection against C. albicans and S. aureus, and has positive implications for controlling diseases mediated by coinfection of these microorganisms (Spellberg et al., 2008).

In summary, polymicrobial infections require ecological and physiological characterization to determine interactomes and changes in target expression based on community characteristics. Therefore, vaccine design for polymicrobial infections should adequately consider the consortia of microorganisms responsible for disease, potential intermicrobial interactions resulting in the modulation of in vivo expressed antigens, and the strategic elimination of microorganisms that enhance or contribute to pathogenesis. Future strategies may be to target vaccination against seemingly nonpathogenic organisms that facilitate increased pathogenicity and colonization of virulent microorganisms. Of course, vaccination against ‘commensals’ may have deleterious immunological and microbiological consequences in the host and will have to be tested rigorously before utilization.

**Considerations for future vaccines: lessons learned from S. aureus**

Effective vaccines are available today for many previously problematic bacterial infections, such as the triple vaccine against C. diphtheriae, C. tetani, Bordetella pertussis (Pichichero et al., 2006), N. meningitidis (Trotter et al., 2008), and S. pneumoniae (Bernatoniene & Finn, 2005). The infections targeted by these vaccines are all mediated by one or a few virulence factors, which, when blocked or otherwise neutralized, prevents pathogenesis. Alternatively, other microorganisms have presented a significant challenge in vaccine development due to a complex disease process and the presence and expression patterns of their respective virulence factors. One such example is S. aureus. This pathogenic species is able to cause a host of different types of infections that are either planktonic (e.g. sepsis and pneumonia), biofilm mediated (e.g. osteomyelitis, endocarditis, chronic skin infections, indwelling medical device infections, chronic rhinosinusitis, dental implantitis, and endophthalmitis), or a combination of both modes of growth (e.g. abscess).

*Staphylococcus aureus* is able to accomplish this array of infections by possessing nearly 70 virulence factors, each with infectious mode-of-growth and time-specific expression patterns. Therefore, the search for a single candidate antigen effective in all these cases has hindered *S. aureus* vaccine development. Additionally, the ability of these vaccines to provide protection against multiple modes of growth, including both planktonic and biofilm infection, has not been addressed adequately. While the suggestion of a prophylactic vaccine against the biofilm mode of growth seems counterintuitive, details emerging about *S. aureus* pathogenicity and modulation of the host immune response support this concept. In addition to the multitude of innate immunity evasion tactics (e.g. inhibition of neutrophil...
chemotaxis, inactivation of complement factors, depletion of leukocyte levels, and inhibition of phagocytosis) (Foster, 2005), in vitro and in vivo studies indicate that S. aureus factors direct the host response toward a beneficial one for the pathogen. In vitro cytokine analyses demonstrate a robust Th1 immune response elicited against S. aureus: staphylococcal enterotoxin B induces IL-2 and IFN-γ (Assemacher et al., 1998), staphylococcal enterotoxin B induces THF-α and MIP-1β (Dauwalder et al., 2006), and whole-cell S. aureus induces IL-12 p70 and IL-18 (Buzas et al., 2004). Studies in a murine model of prosthetic implant infected with S. aureus found upregulation of Th1 cytokines (IL-2, IL-12 p70, and TNF-α) and Th17 cytokines (IL-6 and IL-17) at days 7 and 28 postinfection and increased levels of IgG2b (the dominant Th1-dependent iso-subtype) compared with IgG1 (a Th-2 dependent iso-subtype) in the serum at day 7 postinfection (R. Prabhakara & M. E. Shirliff, unpublished data). These studies indicate that S. aureus elicits a prolonged Th1 response, where the proinflammatory defenses are thwarted by the microbial virulence factors and cause significant damage to the host tissue, and subverts a Th2 humoral response; these skewed immune responses allow the planktonic S. aureus to elude clearance by the immune system as the microorganism colonizes the damaged host tissue and forms a biofilm. Therefore, in order to encompass all aspects of staphylococcal virulence in vaccine development, one must also include an emphasis on biofilms.

Antigen selection: the next generation

In order to correctly select appropriate antigens that will be effective in preventing the establishment of a microbial infection, it is necessary to take into account the planktonic and biofilm modes of growth. Microbial biofilms present a unique challenge to researchers seeking to develop vaccines against microorganisms whose infectivity depends, wholly or in part, on this growth modality. Success cannot be achieved by ignoring the fundamental principle of microbial biofilms: biofilm-resident bacterial cells exhibit a phenotype that is distinct, and in some cases, almost unrecognizable, compared with that of taxonomically identical cells growing planktonically (Beeken et al., 2004; O’May et al., 2009). Thus, both the planktonic and the biofilm phenotype and its implications for antigen expression must be taken into account during the selection of antigens to be included in a vaccine. While the search for a single antigen that provides multimodal protection may prove successful, it seems more likely that a multicomponent vaccine will be necessary. This is the first criterion for an effective broad-range vaccine.

The second is to ensure that the selected antigens are expressed in all relevant strains of the pathogen targeted by the vaccine. The genetic variation of surface-expressed proteins between strains also raises a difficulty. Just such a problem (Thompson et al., 2003; Dyet & Martin, 2005) as well as the structural homology of the polysaccharide capsule with the polysialylated form of the neural cell adhesion molecule (Finne et al., 1983) has held up the development of a broad-range vaccine against type B N. meningitidis, although clinical trials have begun on vaccines developed by reverse vaccinology and other strategies (Granoff, 2010; Sadarangani & Pollard, 2010). For this reason, it is vital to test vaccine efficacy against as large a number of strains as is realistically feasible.

The third principle is to ensure that the candidate antigens are expressed in vivo throughout the infection cycle in the multiple types of infection (e.g. sepsis vs. indwelling medical device infection) for which the pathogen is the identified etiological agent. Once again, like the multiple modes of growth, this protection will most likely need to be accomplished by a multivalent vaccine.

The fourth principle of antigen selection is that either (1) the selected antigen, or (2) the sum of all antigens included in a multicomponent vaccine, must be expressed throughout the infecting microbial population. This is particularly the case when prevention of biofilm-type infections is the goal. Biofilm communities are inherently complex systems, usually existing in close proximity to a surface. This complexity arises from a number of factors. First, distinct physicochemical gradients are found within microbial biofilm communities. In most cases, organic compounds, oxygen, or water enter the biofilm from the surrounding bulk fluid and diffuse through the matrix to the depths closer to the surface. Bacteria resident within a biofilm consume these compounds at varying rates, resulting in differential availability of nutrients, dependent on the location of a particular cell within the community. This effect has been observed experimentally in the case of oxygen tension (de Beer et al., 1994). The situation is further complicated by very low metabolic levels and radically downregulated rates of cell division of the deeply entrenched microorganisms (Brown et al., 1988), including totally nondividing ‘persister’ cells (Harrison et al., 2005; Lewis, 2008). This lowered growth rate is partially responsible for the increased recalcitrance to antimicrobials exhibited by biofilm-embedded bacteria (Gilbert et al., 2002). The end result of this is that cells in different areas of the biofilm exhibit spatial phenotypic heterogeneity, i.e. an antigen expressed by cells in a relatively nutrient-rich area of the community may not be expressed by other cells under less favorable growth conditions. A study by Brady et al. (2006) on S. aureus investigated the ability of polyclonal IgG raised in rabbits against antigens, shown in an earlier work by the same authors to be expressed in S. aureus biofilm in vivo, to visualize S. aureus biofilm communities grown in an in vitro
flow reactor (Brady et al., 2007). Data suggested that although each of the four antigens was expressed within S. aureus biofilm communities, none of them was expressed homogenously throughout the biofilm. Instead, differing expression patterns were observed for each antigen. Hence, inclusion of any one antigen in a monovalent vaccine would likely mean that only a fraction of the biofilm would be targeted and the biofilm would likely survive and the infection would persist. It follows that a multivalent vaccine is essential when prevention of biofilm-type infection is the goal.

Finally, the antigens selected for a biofilm vaccine must be immunologically relevant, meaning that they must be cell-surface proteins that are visible to the humoral immune system and not obscured by the biofilm matrix. Furthermore, each component must be capable of not only eliciting a strong humoral immune response in the host, but a correct response. In some cases, microbial clearance can be promoted by either an inflammatory response (Th1 and/or Th17) or an anti-inflammatory response (Th2 and/or Treg) that can be disease mode, species, or even microbial strain specific. Once again, multivalent vaccines seem to be required to accomplish this principle.

Brady and colleagues used these criteria to select four protein antigens that were demonstrably expressed during S. aureus biofilm growth in vitro, cell-surface associated, and immunogenic in the rabbit model of osteomyelitis (Mader & Shirtliff, 1999; Brady et al., 2007). Singly, combined with the TiterMax™ adjuvant comprised of squalene, sorbitan monooleate 80, and a synthetic block copolymer CRL8941, these antigens were unable to provide protection against S. aureus osteomyelitis in the rabbit model. However, when used together as a prophylactic quadrivalent vaccine (75 µg of each protein administered subcutaneously; one booster 14 days later; both using the TiterMax™ adjuvant) and combined with postinfection vancomycin treatment (5 mg kg⁻¹ twice daily for 10 days) to eliminate planktonic bacteria residing within the bone, eight of nine animals cleared the infection completely. Furthermore, there were significant reductions in radiological and clinical signs of infection in the treated vs. the untreated groups (Brady et al., in press). Research now being conducted is seeking to include S. aureus surface proteins expressed during planktonic growth in order to remove the need for concurrent vancomycin administration.

The unique physiology and properties of biofilm must be taken into account when selecting antigens for inclusion in any vaccine intended to be effective against these communities. Biofilm-type infections can no longer be regarded as merely ‘bacteria embedded within slime’. Biofilm-resident microorganisms are distinct from their free-living counterparts and present unique challenges to anyone seeking to develop novel prophylactic therapeutics.

Conclusions

Vaccine development has primarily focused on the pathogenesis of a single microorganism based on its virulence and immunoavoidance factors and the directed host response to the monomicrobial infection. However, greater appreciation of the fact that many infectious diseases result and persist due to the polymicrobial nature and biofilm maturation of bacteria is challenging many perceptions on vaccine design. Current recombinant vaccines targeting a single or a few bacterial proteins possess the benefits of easy manufacture, no risk of disease from reversion back to a virulent form, and few adverse effects from inflammatory induction compared with whole-cell vaccines. Recombinant vaccine usage does come with the loss of antigen diversity and robust humoral response due to the innate response activation that is provided from vaccination with whole cells. As such, redundancy in bacterial proteins expressed during infection, for example adhesins, subverts responses activated by monovalent vaccines and provides incomplete protection. Antigenic variation has also compelled reassessment of vaccine design due to the observation that in vaccinated individuals the diseases targeted by current clinical vaccines, for example S. pneumoniae 7-valent, shift toward ones acted upon by previously scarce and inconsequential bacterial variants that are not represented in the vaccine (Eskola et al., 2001). Multivalent strategies have come to the forefront in vaccine development in hopes to provide antigenic diversity and sufficient vaccine efficacy, but some clinical trials with multivalent vaccines fail to transition into a later phase, due to the incomplete coverage against disease that is observed.

Staphylococcus aureus-mediated diseases highlight the key properties of the pathogen that are challenges to current vaccine strategies and not appropriately addressed during most vaccine development efforts, including polymicrobial infection, biofilm maturation, and host carrier status. Vaccines targeting S. aureus adherence factors could be ineffective against diseases where coinfecting microorganisms contribute virulence factors in trans and negate the activity of the S. aureus factors, for example hypothetical control of S. aureus adherence by the B. pertussis secreted proteins during coinfection that mimics in vitro findings (Tuomanen, 1986). Once S. aureus colonization is successful and S. aureus immunoavoidance factors obstruct the innate immune response, S. aureus may grow and persist as a biofilm community encapsulated in a polysaccharide matrix. Compounding the problem is that this timed up- and downregulated expression of virulence factors is not only growth phase dependent but also disease specific.

The biofilm phenotype further conceals S. aureus from the immune system due to the downregulated expression of factors that mediate initial infection and encapsulation in polysaccharide that masks surface-associated proteins from
immune recognition. Analysis of the mature *S. aureus* biofilm indicates that there is great heterogeneity in protein expression throughout the biofilm community, with protein expression present in some microcolonies and completely absent in others. As such, a vaccine that targeted these proteins would be ineffective at eliciting an opsonization response to clear *S. aureus*.

Another consideration for vaccine development is the expression of virulence factors that antagonize the immune response, inducing inflammation and tissue damage, where further bacterial colonization can occur; other factors that target and inactivate host immunoglobulins also pose significant problems. Knowledge of the specific immune responses activated by the bacteria and whether that response assists bacterial colonization and persistence will allow the development of vaccines that can modulate the immune response, using adjuvants or extrinsic bacterial components, which skew toward appropriate immunity.

A final consideration for vaccine development is *S. aureus* carriage in humans. Analysis of sera from healthy carriers establishes the circulation of anti-*S. aureus* immunoglobulins, indicating that this response is insufficient to prevent colonization and persistence. Vaccine strategies using antigens targeted by those immunoglobulins will probably elicit a response that is not completely protective. Therefore, screening for and removal of those antigens before protection studies may be advisable. Overall, these properties are critical to understanding how the immune response is ineffective at bacterial clearance. Further evaluation of these features will establish optimal antigenic candidates, including protein factors specific for disease and those not concealed from the immune system that should be established as prerequisites for *S. aureus* and other bacterial vaccines.

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