Phage and Yeast Display

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ABSTRACT Despite the availability of antimicrobial drugs, the continued development of microbial resistance—established through escape mutations and the emergence of resistant strains—limits their clinical utility. The discovery of novel, therapeutic, monoclonal antibodies (mAbs) offers viable clinical alternatives in the treatment and prophylaxis of infectious diseases. Human mAb-based therapies are typically nontoxic in patients and demonstrate high specificity for the intended microbial target. This specificity prevents negative impacts on the patient microbiome and avoids driving the resistance of nontarget species. The in vitro selection of human antibody fragment libraries displayed on phage or yeast surfaces represents a group of well-established technologies capable of generating human mAbs. The advantage of these forms of microbial display is the large repertoire of human antibody fragments present during a single selection campaign. Furthermore, the in vitro selection environments of microbial surface display allow for the rapid isolation of antibodies—and their encoding genes—against infectious pathogens and their toxins that are impractical within in vivo systems, such as murine hybridomas. This article focuses on the technologies of phage display and yeast display, as these strategies relate to the discovery of human mAbs for the treatment and vaccine development of infectious diseases.

DISCOVERY OF THERAPEUTIC ANTIBODIES USING PHAGE DISPLAY TECHNOLOGY

Bacteriophage Biology and Antibody Display Method

Bacteriophage (phage) are viruses that infect and replicate within bacterial cells. Filamentous phage particles inject single-stranded DNA into target bacterial cells for subsequent replication and assembly of new virions within the host cytoplasm. The filamentous phage species capable of infecting Escherichia coli manifest as long, thin filaments that are secreted from host bacteria without cell lysis. Due to their ease of manipulation and stability in a range of temperatures and pH, F pilus-specific filamentous phage species, including f1, fd, and M13, serve as reliable vehicles for combinatorial technologies, such as phage display (1–4).

A collection of the first human antibody libraries displayed on the phage surface was published in the early 1990s (5–8). This antibody phage display technology is based upon a large collection of human antibody genes subcloned into an E. coli expression vector, which is packaged into filamentous phage. Upon production from host bacterial cells, these phage particles display the antibody fragment on its surface as a fusion product with one of the viral coat proteins. This established library must be screened to isolate the phage antibodies of interest. To ensure representation of all immunoglobulin variants within the library, a larger number of phage particles—above the total number of unique antibody sequences—is typically screened. This fold-excess in selection experiments is easily achieved since filamentous phage can produce titers up to 10^{13} particles per ml of culture (1).

The purpose of antibody display library selections is to isolate an antibody variant with the desired functional properties. A purified, heterogeneous population of phage library members is screened to identify and enrich for the antibody sequences of interest. This phage library selection typically involves the capture of the target

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antigen, either by immobilization on a plastic surface (solid phase) or coupled to magnetic beads (solution phase). When incubated with this captured antigen, the phage surfaces—purified virions in solution—allow binding of specific antibody variants. Multiple washes remove nonbinding phage particles, while attached phage are typically eluted under either low- or high-pH conditions to disrupt the antibody-antigen association. Following this elution step, these phage antibodies are used to infect an E. coli culture for amplification of the antigen-reactive library subpopulation. The purified, amplified phage antibodies from this initial selection event are used for subsequent rounds of panning. Due to the possibility of nonspecific binding, at least two to three rounds of this type of iterative library selection are performed to enrich for positive binders and reduce “background” binding events. An overview of this phage library selection procedure is outlined in Fig. 1.

Sources of Antibody Genes for Display Libraries

The quality and diversity of a phage library’s total antibody repertoire is dependent upon the source of...
immunoglobulin genes. To establish the library repertoire, the heterogeneous pool of antibody genes is typically derived from B cell mRNA from healthy donors (naive library) or immunized donors or animals (immune library) (6–7). The variable fragments from the donated B cell cDNA are amplified using germline gene family-based primers (8). These heavy (VH) and light (VL) chain gene copies are randomly associated through cloning methods involving specific expression vectors depending upon the antibody fragment format to be displayed on the phage surface (discussed in “Antibody Phage Display Formats,” below). In addition, synthetic libraries may be established through prearranged V, D, and J gene associations and VH/VL pairings, based upon desired immunogenetic profiles or biochemical properties.

Naive phage antibody libraries
While nonimmunized human B cell donor sources are typically peripheral blood mononuclear cells, other sources of a library’s antibody gene repertoire include spleen, tonsils, bone marrow, and murine peripheral blood mononuclear cells (7, 9–12). The advantage of the naive library lies in the diversity of the antigen-unbiased variants, which can be used for selections against a large panel of targets, including self and toxic antigens (7, 10, 13). The quality of monoclonal antibodies (mAbs) isolated from this type of naive library is dependent upon the total population number, with large, diverse libraries of ∼10^10 unique variants permitting the discovery of human antibodies with subnanomolar binding affinities (10). The availability of large, pre-established collections of nonimmune immunoglobulin gene pools has made the creation of naive phage antibody libraries more convenient than immune libraries (12).

Immune phage antibody libraries
In addition to these naive sources, a phage library’s antibody gene pool may be created from human donors or animals immunized with an antigen of interest. This type of immune library is enriched for antigen-reactive variants with populations of affinity matured VH and VL gene fragments, due to the host immune system machinery (6, 12, 14). Unlike naive gene pools, immune phage libraries are comprised of antibody genes that are biased toward the initial antigen. While this antigenic bias limits the scope of targets for this type of library, immune gene pools are composed of a higher frequency of antigen-reactive library members at the onset of selections. Further, immune phage antibody libraries permit the monitoring of natural immunoglobulin responses, functioning as diagnostics in patients with autoimmune disorders and viral infections (15, 16). Compared with hybridoma campaigns, significantly more mAbs can be isolated from a single immunized donor through an immune phage antibody library (12).

Synthetic phage antibody libraries
An antibody gene repertoire is artificially designed, prior to cloning into a phage expression vector, to create a synthetic phage display library. Usually, the in vitro assembly of novel immunoglobulin genes involves a designed randomization of complementary determining regions (CDRs) into V, D, and J segments (17). The heavy chain CDR3 is considered the most hypervariable of all antigen-contacting loops, causing this region to be targeted for extensive manipulation (18).

Although several optimized phage libraries are established, two examples of modern, synthetic, and semi-synthetic phage display libraries are frequently described in the literature as discovery tools. One corporation created a phage display library that combines both DNA sequences from synthetic design and naive human donors. For this semi-synthetic antibody library, donor-sourced fragments comprise the VH CDR3 and complete VL, while in vitro synthesis of V segment DNA introduces designed diversity in VH CDR1 and CDR2 (19). Another group developed synthetic phage display libraries by modifying the sequence and length of all six CDRs based upon their context in natural human antibody gene families (20–22).

Antibody Phage Display Formats
Previous reports describe phage display libraries as fusion products with all five of the pIII, pVI, pVII, pVIII, and viral coat proteins (5, 13, 23–26). Fusion products of the minor coat pIII—either a truncated or full-length protein—represent the majority of human antibody display libraries. The surface protein pIII is an integral membrane protein that is necessary for phage particle assembly and release from host cells (2). Since only the pIII C-terminus is necessary for new virion construction, a human antibody or antibody fragment can be expressed as a fusion product with this truncated viral protein (3, 5, 13). Schematics of these phage protein-scFv fusions are detailed in Fig. 2. Two of the primary pIII antibody display library types involve human single-chain variable fragment (scFv) or fragment antigen-binding (Fab) formats. The human scFv format consists of a heavy chain variable (VH) domain and light chain variable (VL) domain expressed as a single polypeptide connected by a flexible linker.
region, with no associated constant domains. With Fab libraries, the heavy and light chains are expressed as separate gene products from the same vector with constant heavy 1 and constant light domains, respectively (5). The establishment of modern, optimized, human antibody phage display libraries supports the contemporary role of this combinatorial technology in ongoing antibody discovery (19–22).

**scFv-pIII display**

Within this scFv-pIII display technology, the single-chain antibodies can be expressed as monovalent or multivalent fusion products on the phage surface. The majority of pIII fusion libraries are based on monovalent (phagemid) vectors compared to multivalent (phage) vectors (27). To “rescue” the viral particles from host bacterial cells, the phagemid vector system requires the addition of other wild-type phage genes and proteins via helper phage infection (28). This helper phage infection introduces a wild-type copy of pIII, which outcompetes the scFv-pIII fusion during particle incorporation. This preference for wild-type pIII results in a recovered phage particle population whose majority displays no scFv-pIII fusion on the viral surface, with the second most-frequent population bearing a single copy of the fusion product (27). Despite this wild-type dominance, the monovalent scFv-pIII display technique allows for the selection of higher-affinity antibodies due to single-copy competition among library variants during iterative rounds of selection. This monovalent competition requires at least two to three successive rounds of panning to enrich for scFv-pIII variants over wild-type pIII phage particles.

A multivalent scFv-pIII library can be created through a helper phage population with a gene III deletion, delivery of a helper plasmid, or the subcloning of scFv genes into a true phage vector (13, 27, 29–31). The multivalent display of scFv-pIII fusion proteins permits for the selection of library isolates based upon avidity, rather than affinity. In addition, these phage library-derived variants demonstrate a stronger functional affinity when the target antigen is multivalent, such as the environment during solid phase selections (27). An advantage of this multivalent display strategy is the reduced number of iterative rounds of selection required to isolate antigen-reactive clones. Selection campaigns using a multivalent scFv-pIII library yield antigen-binding populations after only a single round of panning, with antigen-reactive frequencies of >30% observed by the second round (27). Similar selections with monovalent phagemid libraries require at least three rounds of selection before reaching this frequency of positive binding events. Despite this accelerated selection process, multivalent phage library vectors suffer poorer transformation efficiencies, resulting in smaller, less diverse antibody gene pools (27).

**Fab-pIII display**

Early pioneering work in establishing phage display included libraries expressing Fab fragments for selections (5, 9, 32, 33). Modern phage libraries displaying Fab fragments are well established and engineered for improved biochemical and expression properties for therapeutic discovery (19–22). For displaying Fabs on the phage surface, either the heavy or light chain constant domain—preferably the constant heavy 1 to avoid anchored V_{L} dimers—is C-terminally fused to pIII. The nonanchored partner chain is secreted into the periplasmic region, where V_{H}/V_{L} chain pairing occurs to form a complete Fab on the phage surface (12). While the reduced size of the scFv fragment provides for increased genetic stability over Fab libraries, many scFvs are observed to oligomerize, negatively impacting selection strategies (12, 13).

Structural properties of the Fab fragment have desirable features for certain antibody discovery processes. Specifically, the presence of the constant heavy 1 and constant light domains may influence conformational characteristics of the V_{H} and V_{L} fragments. Compared with the scFv format, certain reports have described the

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**FIGURE 2** Types of phage antibody display. (A) Monovalent display with the scFv or Fab fusion (green circle) to truncated pIll along with wild-type copies of pIll (purple circles). This monovalent mAb display format can also be used with pVII (olive) or pIX (light blue) separately. (B) Multivalent display with the scFv or Fab fusion to all copies of truncated pIll. Multivalent mAb display is also possible with the major coat protein pVIII (black border) separate from pIll. The pVI (red circles) coat proteins are also present in these diagrams. doi:10.1128/microbiolspec.AID-0028-2014.f2
increased capacity of selected Fabs to retain antigen specificity when converted to a full-length IgG antibody (34). In addition to favorable biochemical properties, in vivo studies demonstrated the biological potential of Fab antibody fragments. Efficacy studies in a rheumatoid arthritis mouse model reported no significant therapeutic difference between Fab or IgG1 treatments (35). In terms of antibody development, one study described the Fab format as a reliable platform during affinity maturation efforts. The improvements in binding characteristics observed in particular Fab conformations were retained following the conversion to full-length IgG. (36). These findings suggest that the Fab format is a successful antibody fragment surrogate for the selection, prediction, and optimization of IgG candidates.

Antibodies for Infectious Diseases Discovered by Phage Display

The continuing emergence of novel strains and drug-resistant microbial pathogens has created persistent threats to public health. The ongoing discovery of mAbs directed against targets of infectious agents presents clinical opportunities for patients. These antimicrobial mAbs are typically well tolerated in patients and demonstrate high specificity for the targeted pathogen, reducing the opportunity for cross-reactivity with host or nontarget agents.

The in vitro discovery technology of phage display is one of the most promising vehicles for identifying antimicrobial, therapeutic antibodies. This selection method allows for the opportunity to isolate mAbs against toxic species or antigens, a threatening characteristic not suitable for murine hybridoma campaigns. Previous phage display approaches describe the discovery of antiviral and antibacterial mAbs.

Antiviral mAbs discovered by phage display

The efforts of antiviral research are aimed at a fluid pathogenic landscape whose future characteristics are difficult to predict. Among all human pathogens, viruses are the best-suited pathogens to evade host immune pressure. Viral mutational robustness allows for antigenic drift, where escape mutations within targeted epitopes result in progeny with increased resistance to neutralizing mAbs or small molecules (37). Furthermore, coinfections with two or more viral strains allow for antigenic shift through gene transfer, establishing novel pathogens and complicating vaccine and treatment strategies (38–40). These continuous modes of viral evolution necessitate the development of new treatment options, which include the discovery of antiviral mAbs.

The targets of early antiviral mAb discovery using phage display included human immunodeficiency virus type 1 (HIV-1), respiratory syncytial virus, and herpes simplex virus (14, 16). Surface viral glycoproteins are preferred targets for phage selection campaigns, given their extracellular exposure, typically soluble character, and critical roles in the viral life cycle (41). Viral neutralization is a significant trait under consideration during the characterization of mAbs isolated from selections against viral antigens. This capacity of antiviral mAbs to bind and inactivate viral particles is initially evaluated through designed in vitro experiments. Following these primary bioassays, the in vivo protection of lead candidate mAbs is determined through viral challenge studies in animals. Potential mechanisms of viral neutralization include the inhibition of virus attachment to host cell receptors, the blockage of membrane fusion events releasing viral genetic material, and prevention of new virion release from infected cells (42, 43).

Outside of direct inactivation, nonneutralizing antiviral mAbs may bind viral antigens on the cell surface during progeny release, recruiting cytotoxic cells or complement proteins to offer in vivo protection. These findings fueled the ongoing debate surrounding the correlation between in vitro neutralization and in vivo protection for antibodies (41, 44). Since the principal fraction of nonneutralizing mAbs fail to clear viral infections in vivo, however, the emphasis during antiviral mAb discovery should be placed on neutralizing immunoglobulins (41). A brief summary of antiviral mAbs discovered using phage antibody libraries is listed in Table 1.

Anti-influenza mAbs discovered by phage display

One of the most popular targets for recent antiviral mAb discovery is the influenza virus. Due to the absence of a universal vaccine and the ongoing emergence of new strains as human pathogens, the discovery of protective mAbs against influenza remains relevant. Influenza A viruses—the group of influenza viruses most closely associated with human infections—are classified within the classical subtypes of H1-H16 and N1-N9 based upon structural and antigenic characteristics of the hemagglutinin (HA) and neuraminidase viral glycoproteins (45). The HA molecule is the major surface envelope protein and is responsible for mediating attachment to sialic acid on the target cell surface (46). The globular head of HA, which contains the sialic acid binding domain, is amenable to mutations to avoid immune pressure without significant costs to viral fitness (41, 47). The HA stem domain is involved in the
Although these discoveries provided initial data, selection antigen have also identified mechanisms to avoid neutralizing mAbs, including masking of critical epitopes, amino acid mutations, and glycan shielding without loss of protein activity (48).

Seminal phage display studies using purified, recombinant HA protein as the selection antigen reported whole panels of broadly neutralizing human mAbs (49–51). The broadly neutralizing mAbs isolated from these phage library selections are overwhelmingly biased toward the conserved stem domain, preventing endosomal membrane fusion but permitting viral attachment. Given the stem region conservation and epitope proximity, the potent neutralization among these phage display-selected mAbs is observed across multiple subtypes of influenza viruses. Following up on these initial studies, selections using a semi-synthetic phage library identified specific immunoglobulin signatures associated with heterosubtypic neutralization for stem-directed anti-HA mAbs (52).

Phage display experiments using influenza HA as the selection antigen have also identified neutralizing antibodies directed against the globular head of the glycoprotein. While these antibodies are capable of binding HA proteins across multiple viral subtypes, globular head-directed mAbs tend to be less heteroreactive, given the lack of strict conservation among these epitopes. Studies involving phage library selections yielded head-directed mAbs capable of neutralizing single or multiple subtypes, including H1N1, H2N2, and H3N2 viruses (53–54). Despite this limited scope of reactivity, mAbs that are neutralizing to the globular head are typically more potent, a trend likely due to epitope availability on the viral surface (41). Neutralization escape variants are more easily generated by immune pressure on the globular head, however, as the loops and glycosylation sites allow this region to tolerate mutations (55).

**Anti-HIV-1 mAbs discovered by phage display**

Another well-documented target for phage display library selections is HIV-1. The primary target of anti-HIV-1 humoral responses is the trimeric Env protein of the viral surface. The gp120 protein of this Env complex binds the CD4 molecule and the CCR5 or CXCR5 co-receptors on CD4+ T cells and macrophages. This engagement causes a conformational change in the Env complex to induce the membrane fusion activity of the gp41 component (56). One of the primary challenges in developing an HIV-1 vaccine strategy or broadly neutralizing mAbs is the extensive genetic diversity, with up to 10% Env gene variability within a single patient (57). Similar to influenza HA, the HIV-1 Env trimer utilizes several mechanisms to avoid neutralizing mAbs, including masking of critical epitopes, amino acid mutations, and glycan shielding without loss of protein activity (58).

Among the collection of first-generation anti-HIV-1 mAbs, several neutralizing library variants, including mAb b12, were isolated using phage display technology (59, 60). Although these discoveries provided initial data related to the CD4 binding site epitope, these mAbs are limited in their scope of neutralization, with mAb b12 neutralizing only one third of total HIV-1 isolates (41). More recent phage library selections against the viral gp120 and gp41 antigen yielded mAbs neutralizing a

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**TABLE 1** Summary of antiviral antibodies discovered using phage display

<table>
<thead>
<tr>
<th>Viral family</th>
<th>Virus</th>
<th>Viral target</th>
<th>Ab fragment displayed</th>
<th>Ab source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronaviridae</td>
<td>MERS-CoV</td>
<td>Spike glycoprotein</td>
<td>scFv</td>
<td>Naive</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>MERS-CoV</td>
<td>Spike receptor binding domain (RBD)</td>
<td>Fab</td>
<td>Naive</td>
<td>73</td>
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<td></td>
<td>SARS-CoV</td>
<td>Spike, S1 domain</td>
<td>scFv</td>
<td>Naive</td>
<td>65</td>
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<tr>
<td></td>
<td>SARS-CoV</td>
<td>Spike RBD</td>
<td>Fab</td>
<td>Naive</td>
<td>66</td>
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<tr>
<td>Filoviridae</td>
<td>Ebola virus</td>
<td>Inactivated virus</td>
<td>Fab</td>
<td>Immune</td>
<td>78</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Dengue virus</td>
<td>mAb-captured dengue virus</td>
<td>scFv</td>
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<td>76</td>
</tr>
<tr>
<td></td>
<td>Dengue virus</td>
<td>Nonstructural protein S (NS5)</td>
<td>Fab</td>
<td>Naive</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>West Nile virus</td>
<td>E glycoprotein</td>
<td>scFv</td>
<td>Naive</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>West Nile virus</td>
<td>Inactivated virus, VLP, or E glycoprotein</td>
<td>scFv</td>
<td>Immune</td>
<td>75</td>
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<tr>
<td>Herpesviridae</td>
<td>Herpes simplex virus</td>
<td>mAb-captured gB and gD glycoproteins</td>
<td>Fab</td>
<td>Immune</td>
<td>81</td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>Influenza A virus</td>
<td>Hemagglutinin (HA)</td>
<td>scFv</td>
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<td>49–51</td>
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<tr>
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<td>Influenza A virus</td>
<td>HA</td>
<td>scFv</td>
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<tr>
<td></td>
<td>Influenza A virus</td>
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<td>scFv</td>
<td>Immune</td>
<td>53</td>
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<tr>
<td></td>
<td>Influenza A and B viruses</td>
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<td>scFv</td>
<td>Immune</td>
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<td>Fab</td>
<td>Immune</td>
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<td>gp41</td>
<td>Fab</td>
<td>Immune</td>
<td>81–82</td>
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*Note: This list is a summary of selected publications and is not to be considered complete.*
diverse collection of primary isolates and subtypes (61–63). In addition, the immunization schedule using HIV-1 antigens influences the selection quality of anti-HIV-1 mAbs from immune phage libraries, consistent with earlier findings regarding immune libraries’ capacity for tracking natural humoral responses in humans (64). While these phage display-related studies offered primary mapping data and early vaccine strategies, current anti-HIV-1 mAb discovery efforts rely more heavily on single B cell sorting or reverse transcriptase PCR techniques and the screening of in vitro-activated B cells (41).

Anticoronavirus mAbs discovered by phage display

The severe acute respiratory syndrome coronavirus (SARS-CoV) predated its Middle East respiratory syndrome coronavirus (MERS-CoV) relative as an emerging human pathogen during the early 2000s. A potent neutralizing antibody was identified from panning experiments with purified, truncated S1 domain-only antigen (65). Other selection studies produced a similarly neutralizing mAb (66). These phage display studies using SARS-CoV antigen fragments influenced the future strategies surrounding the current MERS-CoV emerging infectious disease.

A human pathogen recently targeted by phage display studies is the emerging MERS-CoV. Similar to its SARS-CoV Coronaviridae family member, the MERS-CoV strains are capable of residing in other mammalian species, including bats and camels (67, 68). The MERS-CoV uses the major envelope spike (S) glycoprotein trimer for attachment and entry into target cells, with the S1 domain containing the dipeptidyl peptidase 4 (DPP4) receptor binding site and the S2 region mediating membrane fusion (69, 70). Previous reports involving infected patients describe the production of S protein-directed neutralizing antibodies (71).

Recently published studies describe phage library selections to identify panels of neutralizing mAbs. Selections against full-length MERS-CoV S protein using scFv-pIII display libraries yielded neutralizing antibodies localizing to the receptor binding domain. Despite the presence of the full-length protein during selections, this campaign resulted in S1 domain-specific mAbs, which neutralized MERS-CoV pseudotyped virus-like particles by blocking the DPP4 binding site on the viral glycoprotein (72). Similarly, Fab-pIII library selections against truncated DPP4 receptor binding domain-only antigen yielded mAbs capable of neutralizing both pseudotyped and live MERS-CoV (73). These studies provide examples of the ongoing relevance of phage display technology in current, emerging infectious diseases.

Other antiviral mAbs discovered by phage display

In addition to the viruses discussed above, mAbs directed against other viral pathogens are detailed in the literature. Initial studies involving phage library selections against purified West Nile Virus (WNV) envelope E glycoprotein produced neutralizing antibody sequences (74). Additional neutralizing mAbs were isolated from phage library selections using inactivated WNV or virus-like particles as well as immobilized E glycoprotein (75). A total of nine unique antibody sequences were identified from phage library selections against Ab-captured dengue virus particles; all members of this mAb panel demonstrated some degree of cross-reactivity among the four dengue virus serotypes (76). Recent phage library panning experiments using purified dengue nonstructural protein 5 generated a collection of cross-reactive mAbs intended for future mechanistic studies of the viral replication machinery (77).

Outside of flaviviruses and coronaviruses, other viral pathogens previously served as targets of early phage display campaigns. Experiments involving inactivated Ebola virus isolates generated one antiglycoprotein mAb capable of neutralizing the virus in vitro (78). Neutralizing mAbs showing in vivo protection against respiratory syncytial virus were isolated from preliminary Fab library selections against the viral F glycoprotein (79–80). Similarly, neutralizing mAbs preventing the cell-to-cell transmission of herpes simplex virus were discovered from pannings against glycoprotein D (81).

Antibacterial mAbs discovered by phage display

The ongoing development of bacterial resistance to antimicrobial agents continues to pose global threats to both healthy individuals and infected patients. Specifically, the misuse of antimicrobial drugs drives bacterial evolution such that antibiotics are becoming increasingly ineffective among Gram-negative and Gram-positive strains (82–85). Previous clinical observations support the role of passive immunotherapy as prophylactic and therapeutic strategies against bacterial pathogens and their toxins (86).

Phage display technologies allow for antibody library selections against bacterial antigens, infectious agents which are impractical for in vivo approaches such as hybridoma campaigns. Unlike approaches with viral pathogens, successful phage display efforts surrounding antibacterial mAb discovery involve secreted toxins as selection antigens, rather than surface or structural proteins. These species-specific exotoxins cause patient symptoms including diarrhea, renal failure, paralysis, and death (87).
In terms of these secreted toxins, mAb-based antibacterial therapies offer several advantages over antibiotic compounds. These antibiotic drugs do not demonstrate the ability to eliminate pro-inflammatory bacterial factors or cell fragments, which can be neutralized or cleared through mAb binding. This outcome may permit disease progression despite the removal of the bacterial pathogen. Further, toxin neutralization mechanisms create more feasible preclinical models for evaluating novel mAbs, accelerating their discovery (87). A brief summary of antibacterial mAbs discovered using phage antibody libraries is listed in Table 2.

**Antibotulinum mAbs discovered by phage display**
The *Clostridium* genus encompasses a group of Gram-positive bacteria species capable of secreting toxins. *Clostridium botulinum* produces botulinum neurotoxins (BoNTs) of serotypes A-G, with the A, B, and E serotypes most frequently causing human botulism (88). These BoNTs act at cholinergic nerve endings to prevent acetylcholine release, providing the paralytic effects of these exotoxins (89). Phage library selections against BoNT serotypes A-E produced neutralizing mAbs, with the successful clones preferentially isolated from immune sources (88, 90). The most potent of these neutralizing mAbs were directed against the BoNT heavy chain C-terminus. The administration of any mAb pair recognizing nonoverlapping heavy chain epitopes provided complete protection during murine in vivo toxin neutralization experiments, while single antibody delivery resulted in significant viability loss (91). The ongoing mAb discovery surrounding BoNTs remains important, given their classification as high-risk agents as bioweapons (91).

**Antianthrax mAbs discovered by phage display**
Similar to the *C. botulinum* BoNTs, the Gram-positive *Bacillus anthracis*, the causative agent of anthrax, is considered a public safety threat because of its use as a biological weapon (92). Upon establishing an infection within lymph nodes, dividing bacilli release three exotoxins: protective antigen (PA), lethal factor (LF), and edema factor (EF). The PA toxin binds cellular receptors for delivery of LF or EF into the host cytosol. The PA toxin may combine with LF to form lethal toxin or EF to create the edema toxin, both of which induce the toxic symptoms associated with anthrax disease (93).

Phage library selections against recombinant PA resulted in the discovery of raxibacumab (ABthrax), a human IgG1 mAb that binds PA to inhibit cellular delivery of LF and EF to prevent and treat inhalational anthrax (94). Prior to FDA approval, this anti-PA mAb demonstrated in vivo protection in both rabbits and monkeys. An anti-LF scFv produced from phage library panning demonstrated in vitro toxin neutralization and in vivo protection (95). Phage library selections against recombinant EF yielded an anti-EF mAb that protected mice from edema and death during lethal dose administration (96). These phage display studies continue to be valuable in defining protective, biodefense strategies.

**Other antibacterial mAbs discovered by phage display**
Bacterial pathogens other than *C. botulinum* and *B. anthracis* are described as targets of phage display experiments. A relative of *C. botulinum*, the *Clostridium difficile* pathogen is the leading cause of antibiotic-associated diarrhea, with increasing severity due to the ongoing emergence of hypervirulent strains (87). Disease onset occurs by production of toxins A and B following intestinal colonization. Phage library panning experiments against *C. difficile* toxin B generated a mAb, with improved affinity over the parent antibody, intended for use as a novel immunodiagnostic agent (97). *Clostridium tetani*, another member of this *Clostridium* genus,

<table>
<thead>
<tr>
<th>Bacterial family</th>
<th>Bacterial species</th>
<th>Bacterial target</th>
<th>Ab fragment displayed</th>
<th>Ab source</th>
<th>Reference</th>
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<tr>
<td>Bacillaceae</td>
<td><em>Bacillus anthracis</em></td>
<td>Protective antigen</td>
<td>scFv</td>
<td>Naïve</td>
<td>94</td>
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<td></td>
<td><em>Bacillus anthracis</em></td>
<td>Lethal factor</td>
<td>scFv</td>
<td>Immune</td>
<td>95</td>
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<tr>
<td></td>
<td><em>Bacillus anthracis</em></td>
<td>Edema factor</td>
<td>Fab</td>
<td>Immune</td>
<td>96</td>
</tr>
<tr>
<td>Clostridiaceae</td>
<td><em>Clostridium botulinum</em></td>
<td>Botulinum neurotoxin (BoNT) subtypes A–E</td>
<td>scFv</td>
<td>Immune and naïve</td>
<td>88</td>
</tr>
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<td></td>
<td><em>Clostridium botulinum</em></td>
<td>BoNT/A heavy chain</td>
<td>scFv</td>
<td>Immune</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium difficile</em></td>
<td>Toxin B</td>
<td>scFv</td>
<td>Immune</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium tetani</em></td>
<td>Tetaus toxoid</td>
<td>Fab</td>
<td>Naïve</td>
<td>98</td>
</tr>
<tr>
<td>Staphylococcaceae</td>
<td><em>Staphylococcus aureus</em></td>
<td>GrfA peptide fragments</td>
<td>scFv</td>
<td>Immune</td>
<td>99</td>
</tr>
</tbody>
</table>

*Note: This list is a summary of selected publications and is not to be considered complete.*
establishes infection through open wounds and secretes tetanus toxoid to induce painful muscle rigidity and spasms. A mAb isolated from library selections against tetanus toxoid demonstrated in vitro neutralization of this exotoxin (98).

While the majority of successful phage library panning campaigns involve exotoxins, less successful experiments involving bacterial surface proteins are described in the literature. *Staphylococcus aureus* continues to present major challenges in clinical settings due to the tendency of this species to develop antibiotic resistance, such as methicillin-resistant *S. aureus* (MRSA). Phage library selections against peptide fragments of the GrfA surface macromolecule transporter from MRSA resulted in a lead candidate scFv (Aurograb), which eventually failed in clinical trials (87, 88). Given the spread of MRSA strains among hospitals, its elevated resistance rate to antibiotics, and the current failure of available human antibodies, the continued development of new mAb selection strategies will create improved therapeutic alternatives during patient infections.

**DISCOVERY OF THERAPEUTIC ANTIBODIES USING YEAST DISPLAY TECHNOLOGY**

**Yeast Biology and Antibody Display System**

Yeast are eukaryotic, unicellular organisms comprising a variety of strains belonging to the kingdom *Fungi*. The budding yeast *Saccharomyces cerevisiae* possesses a rigid cell wall approximately 200 nm thick, primarily composed of mannoproteins and β-linked glucans (100). The budding yeast cell wall surface agglutinins function as adhesion proteins, promoting aggregation of opposite mating types during mating events (101). The Aga2p subunit, which binds the agglutinin proteins of the opposite mating type, is tethered to the yeast cell wall through two disulfide bonds to Aga1p (100). The cell wall of each yeast cell can display $10^4$ to $10^5$ agglutinins (100, 102). Since the development of the yeast display technology, *S. cerevisiae* has served as the most commonly used species in this combinatorial strategy.

To establish a yeast antibody display library, the heterogeneous immunoglobulin gene pool is cloned into the yeast display plasmid and expressed as fusion products with the Aga2p protein. The human antibody fragments are typically fused to the C-terminus of the Aga2p subunit (100). While the scFv is the most frequently used format in yeast display, other antibody fragments may be displayed on the yeast surface, including Fabs, whole IgG1s, and camelid domain antibodies (102).

The Aga1p cell wall protein is stably expressed from a chromosomal locus. The expression of both Aga1p and the mAb-Aga2p product are under the control of the galactose-inducible GAL1 promoter (102, 103). A diagram detailing the yeast surface display system is shown in Fig. 3.

In the presence of galactose, the yeast cell displays the antibody fragment fused to the C-terminus of the Aga2p subunit. Variations in the surface density of this combinatorial complex can be monitored and normalized using immunofluorescent labeling of either the HA or c-Myc tags bordering the scFv or Fab. Since protein expression is induced following cell growth, the GAL1 promoter protects potentially toxic antibody fragments from negative selection. Following the subcloning of the antibody gene repertoire into the yeast display vector, the typical established library consists of $10^7$ to $10^9$ unique variants (100, 103).

Although the early studies with yeast display predominantly involved affinity maturation of existing antibody sequences, recent discovery campaigns show the successful isolation of novel, *de novo* immunoglobulins. Both magnetic assisted cell sorting (MACS) and fluorescence activated cell sorting (FACS) procedures are employed during isolation of antibody clones from yeast libraries (103). Due to the flow cytometry rate limitations of $10^7$ to $10^8$ cells per hour, the MACS steps are used to remove nonbinding mAb fragments to reduce the cell input number for subsequent FACS selection. These MACS separations mimic the panning process from phage display to remove nonbinding yeast cells. Even less successful MACS selections can reduce the nonreactive background by approximately 100 times, permitting reasonable yeast cell numbers for FACS screening (102).

Following this MACS-based depletion, the antigen-reactive population is screened by FACS to enrich for candidate clones. The antigen concentration during yeast library screening is kept at 10-fold excess above the desired dissociation constant ($K_D$) of individual clones to allow the majority of the displayed mAb fragments to be engaged at equilibrium. Further, this antigen excess prevents the titration of target by antibody binding (102). These MACS-selected yeast cells are labeled for the simultaneous detection of scFv or Fab expression (using the c-Myc tag) and antigen binding (using the biotin label on this target). During this first round of FACS selection, the top 5% of this double-positive population is typically collected to protect diversity, with the selection gate narrowed to the top 0.1 to 1% in subsequent rounds of flow cytometry screening (103).
Following three to five rounds of these FACS selections, the plasmid DNA is isolated from this heterogeneous, sorted population and used to transform competent *E. coli* cells. Plasmid DNA is isolated from a selection of these *E. coli* transformants for antibody sequence analysis.

**Yeast Display versus Phage Display Technologies**

Both advantages and drawbacks exist when comparing yeast and phage antibody library technologies, although their gene repertoires may arise from the same sources (see “Sources of Antibody Genes for Display Libraries,” above). The most immediate advantage of yeast display is the accurate control over selection parameters during FACS screening. The collected population percentage, signal normalization, and desired binding affinities can be fixed by the flow cytometry boundaries. This ability to define binding criteria during the selection process represents an advantage over phage display platforms, where variant discrimination is dependent on washing steps rather than real-time kinetic observations (102). In other words, this systematic bias toward desired binding properties available during yeast library selections is not available during phage library campaigns.

Other advantages surrounding yeast display technologies are rooted in the quality of antibody fragment displayed. Yeast libraries make use of the eukaryotic, posttranslational modifications to display scFvs or Fabs in a similar fashion as mammalian cells. Yeast cells employ similar glycosylation patterns as mammalian systems, improving antibody solubility as well as removing a future unknown attribute during full-length IgG expression in mammalian systems. In addition, proper folding events within the yeast endoplasmic reticulum, in the presence of chaperone proteins, protect library diversity by permitting expression of clones too complex for prokaryotic processing used by *E. coli* cells during phage display (102).

While this eukaryotic translation machinery offers expression advantages over phage display libraries, certain drawbacks exist in yeast display approaches. The total number of unique antibody clones in yeast libraries is always several orders of magnitude lower than phage libraries, due to the lower transformation efficiency observed in yeast cells. Yeast antibody libraries possess a theoretical limit of $1 \times 10^7$ to $1 \times 10^9$ total clones, with phage display libraries capable of representing $1 \times 10^6$ to $1 \times 10^{11}$ variants (103). However, recent advances in transformation optimization allow for the
routine generation of yeast antibody display libraries with $1 \times 10^{10}$ total unique clones (104).

Another disadvantage of the yeast display system involves the number of antibody copies displayed per cell. Since each yeast cell displays $1 \times 10^4$ to $1 \times 10^5$ scFv or Fab copies, the selection events are based upon antibody avidity, rather than affinity. This property is emphasized during selections against oligomeric antigens, where the multicopy yeast display profile allows for multivalent binding (102). This antibody density on the yeast cell surface may result in the isolation of variants with lower affinity than those discovered from monovalent phage display methods.

A modern selection strategy makes use of both phage and yeast selections. In this study, two rounds of phage selections against *Mycobacterium tuberculosis* Ag85 were performed. Following this naive phage library selection, this antigen-enriched population was subcloned into a yeast display system for mini-library construction, which yielded over 100 unique mAbs (105). This combined selection approach takes advantage of the strengths of both technologies: the large library size and affinity-based selections of phage display and the controlled, defined collection of variants from yeast display. This powerful, two-system strategy should be considered in future discovery efforts, given the larger panel of valuable mAbs isolated through the strengths of both technologies.

**Antibodies for Infectious Diseases Discovered by Yeast Display**

Although the previously discussed aspects of yeast display highlight the capacity of this combinatorial technology as an effective selection system, the use of yeast libraries for novel antibody discovery is relatively new compared with the history of phage display libraries. Since the initial application of yeast display technologies focused on epitope mapping and affinity maturation, the current employment of yeast surface display in the field of antibody discovery for infectious disease remains biased toward these structural and functional characterization studies (102). Compared to phage display approaches, the published list of antiviral and antibacterial mAbs isolated from yeast libraries is limited.

**Antiviral mAb discovery involving yeast display**

Ongoing research surrounding the discovery of novel antiviral mAbs is supported by the yeast display technology. Unlike phage display, epitope mapping serves as the most popular application of yeast libraries in the development of antiviral mAbs. Typically, mAbs isolated from phage libraries or murine hybridoma campaigns are mapped to specific epitopes on the target antigen by yeast display techniques. However, certain reports describe the use of yeast library selections for the discovery of novel antiviral mAbs. Despite this efficacy, fewer antiviral mAb discovery efforts using yeast display have been published. The most common viral targets for yeast display studies include HIV-1 and flaviviruses. As this yeast display platform continues to develop, additional viral pathogens should be targeted using yeast antibody libraries. A brief summary of antiviral mAb discovery efforts using yeast antibody libraries is listed in Table 3.

**Anti-HIV-1 mAbs discovered by yeast display**

Separate selection campaigns against recombinant HIV-1 gp120 using yeast and phage display systems provided a comparative study examining the collection of selected antibodies. These libraries were created from the same immune donor pool and screened using the same antigen, limiting the investigation to display system differences (106). Yeast library selections produced at least twice as many unique antibody variants as the phage selections, including all the mAbs identified from the phage library. The authors attributed this difference in antibody recovery to the eukaryotic expression of the scFv fragment as the primary factor between yeast and phage display. This increased sensitivity in selecting against gp120 supports the demand for future yeast library technology in antiviral antibody isolation.

**Anti-flavivirus mAb discovery involving yeast display**

Among the flaviviruses, WNV is targeted for protective antibody discovery. Antibody-mediated neutralization of WNV involves recognition of the E glycoprotein. This WNV surface E protein is characterized by three separate structural domains. Yeast display experiments using hybridoma-derived mAbs provided the molecular basis for their neutralization activity (107). The majority of the mAbs characterized in this study localized to domain III (DIII) of the viral E antigen. In addition, yeast surface display mapped a panel of E-selected neutralizing mAbs from a phage library to specific domains of the viral glycoprotein (72). These phage library-derived scFvs selectively bound DI/DII, with no interaction with DIII. These yeast-library mapped variations in E glycoprotein epitope localization may be rooted in the different antigen selection methods. These yeast display-detailed protective epitopes are projected to influence ongoing vaccine strategies against WNV.
Similar to WNV, the four serotypes of dengue virus (DENV1-4) possess a surface E glycoprotein composed of three distinct domains. Yeast display strategies using a series of mutant DIII fragments from the DENV-1 E protein revealed the single or combinations of residues for the binding of seven mAbs isolated from murine hybridomas (108). Likewise, panels of neutralizing mAbs against DENV-2, DENV-3, and DENV-4 were finely mapped across all three domains of the E antigen using yeast libraries (109–111). Recent selections isolated human antibodies against dengue E protein from yeast display libraries (112). These novel, competitive yeast library selections were based upon cross-reactive, neutralizing epitope determinants provided from yeast mapping studies (109). Competitive FACS sorting in the presence of a mutant DIII fragment expressed as an Fc fusion protein produced several wild-type DIII-Fc-reactive populations. The observation of these wild-type antigen-binding subpopulations suggests the mutant protein competition influenced yeast library selections. This unique strategy of competing antigens during selections offers a novel method for isolating epitope-defined variants from yeast antibody display libraries.

**Anti-influenza mAb discovery involving yeast display**

Previous fine epitope mapping studies for anti-influenza mAbs describe the use of yeast display libraries. Broad domain mapping for independent panels of anti-influenza mAbs are described from yeast libraries displaying full-length, precursor influenza HA (HA0) or the HA1 and HA2 subunits separately (113, 114). After confirming binding and domain localization, sublibraries of mutant HA fragments were generated by error-prone PCR for fine epitope mapping. Loss of binding to particular mutant HA subunits indicated particular residues on the viral envelope protein required for mAb binding. These investigations provided structural neutralization determinants for separate mAb panels against surface HA from high-pathogenic influenza strains.

**Antibacterial mAb discovery involving yeast display**

The advantages surrounding yeast surface display for antibacterial mAb discovery offer the same strengths as phage libraries, namely the elimination of in vivo obstacles imposed by hybridoma campaigns and the rapid, high throughput selection of whole antibody libraries. Similar to the discovery work with phage libraries, the primary success with yeast library selections involves soluble, secreted exotoxins, rather than bacterial cell wall–or membrane-associated antigens. In keeping with the trends discussed concerning antiviral mAb discovery, the majority of published yeast library studies describe epitope mapping or affinity maturation. While recent studies describe success with novel antibacterial mAb isolation from yeast libraries, the shorter history of these technologies results in a shorter catalog of published discovery data. A brief summary of bacterial mAb discovery efforts using yeast antibody libraries is listed in Table 4.

**Antibotulinum mAb discovery involving yeast display**

Recent selection efforts using yeast display libraries report the successful discovery of novel mAbs binding

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**TABLE 3** Summary of antiviral antibodies discovered using yeast display

<table>
<thead>
<tr>
<th>Viral family</th>
<th>Virus</th>
<th>Viral target</th>
<th>Ab fragment or Ag displayed</th>
<th>Ab source</th>
<th>Library application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaviviridae</td>
<td>Dengue virus (DENV)</td>
<td>DENV-1 E glycoprotein</td>
<td>DENV-1 E DIII mutants</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>Dengue virus</td>
<td>DENV-2 E glycoprotein</td>
<td>DENV-2 E DI-DIII mutants</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Dengue virus</td>
<td>DENV-3 E glycoprotein</td>
<td>DENV-3 E wild-type and mutant DIII fragments</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Dengue virus</td>
<td>DENV-4 E glycoprotein</td>
<td>DENV-4 E ectodomain and DI-DIII fragments</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Dengue virus West Nile virus</td>
<td>DENV DIII fragments</td>
<td>Fab</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>E glycoprotein</td>
<td>WNV E ectodomain and mutant DIII fragments</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td></td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>Influenza A virus</td>
<td>HA</td>
<td>Wild-type HA0, HA1, and HA2 subunits; HA1 subunit mutants</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retroviridae</td>
<td>HIV-1 gp120 scFv</td>
<td>Immune</td>
<td>mAb selections</td>
<td>106</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: This list is a summary of selected publications and is not to be considered complete.
TABLE 4 Summary of antibacterial antibodies discovered using yeast display

<table>
<thead>
<tr>
<th>Bacterial family</th>
<th>Bacterial species</th>
<th>Bacterial target</th>
<th>Ab fragment or Ag displayed</th>
<th>Ab source</th>
<th>Library application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillaceae</td>
<td><em>Bacillus anthracis</em></td>
<td>Edema factor</td>
<td>Mutant EF DIII fragments</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td><em>Bacillus anthracis</em></td>
<td>Protective antigen (PA)</td>
<td>Mutant N-terminal PA fragments (PA20)</td>
<td>N/A</td>
<td>Epitope mapping</td>
<td>119</td>
</tr>
<tr>
<td>Clostridaceae</td>
<td><em>Clostridium botulinum</em></td>
<td>Botulinum neurotoxin (BoNT) subtype A</td>
<td>scFv</td>
<td>Naive</td>
<td>mAb selections</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium botulinum</em></td>
<td>BoNT subtypes B, E, and F</td>
<td>Fab</td>
<td>Immune</td>
<td>Affinity maturation</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td><em>Clostridium botulinum</em></td>
<td>BoNT subtypes A and B</td>
<td>scFv and Fab</td>
<td>Immune</td>
<td>Affinity selections and mAb selections</td>
<td>117</td>
</tr>
</tbody>
</table>

*Note: This list is a summary of selected publications and is not to be considered complete.

Antianthrax mAb discovery involving yeast display

Fine epitope mapping studies using yeast surface display provided the structural requirements for mAbs neutralizing *B. anthracis* toxin components. The screening of a yeast display library composed of mutant edema factor DIII fragments identified critical residues for neutralization of an anthrax toxin (118). These yeast display efforts revealed that the heavy chain CDR3 of an EF-neutralizing mAb competes for an epitope shared with calmodulin, which serves as an enzymatic activator of *B. anthracis* edema factor. In the same fashion, a yeast library displaying mutants of the N-terminal region of the protective antigen (PA20) defined the contact residues of a known, neutralizing mAb (119). These mapping data indicated that the published mAb prevents the furin-mediated activation of PA, despite the contact residues being distant from the furin recognition sequence.

**CONCLUSIONS**

This article details the development and use of the phage and yeast display technologies for the discovery of novel mAbs for infectious diseases. Through the application of these combinatorial methods, the successful isolation of unique antibodies as treatment options and vaccine development tools continues to be reported. The discovery of pathogen-specific mAbs alleviates the clinical challenges posed by the enduring development of microbial resistance, which is provided through escape mutations and evolution of resistant strains. These isolated mAbs offer nontoxic, target-specific treatment options and vaccine strategy determinants for viral and bacterial pathogens.

The selection of high-affinity (within and below the nanomolar range) antibodies across formats (scFv and Fab) and degrees of valency (monovalent and multivalent) describes the flexibility of the phage and yeast display approaches. The continued improvement of phage library quality has maintained the relevance of this discovery platform for today’s emerging infectious diseases. In addition, the ongoing success of antibody selections using yeast display libraries drives the expansion of this approach beyond its early epitope mapping and affinity maturation applications. These microbial surface display technologies offer powerful platforms for antibody drug discovery and vaccine development within and beyond infectious diseases.

**ACKNOWLEDGMENT**

Conflicts of interest: We declare no conflicts.

**REFERENCES**


Phage and Yeast Display


