

Virus Vectors (Phage Vectors):

Phage Basics

Bacteriophage: Overview and Applications in Molecular Biology

References: Barrow & Soothill. Trends Microbiol. 5:268, 1997 (phage therapy)

Smith & Scott. Methods Enzymol. 217:228, 1993 (phage display methods)

Russell SJ. Nature Medicine 2:276, 1996 (phage display & gene targeting)

Phage Basics

Bacteriophages were first described by Frederick Twort in 1915 and Felix d'Hérelle in 1917. D'Hérelle named them bacteriophages because they could lyse bacteria on the surface of agar plates (phage: from the Greek, "to eat"). There turn out to be a wide variety of phages with different shapes, host ranges and genetic composition -- much like mammalian viruses. Phage research in the 1930s thru 1950s led to the discovery of a large number of key concepts in biology and virology. For example, the Hershey and Chase experiments which established that viral nucleic acid carries the genetic information were performed in the phage system in the mid 1940s.

Temperate phages: Many phages kill their hosts (**lysis**), but in some cases the host can survive the infection, while also stably inheriting the genetic information of the infecting phage. Such bacterial strains can lyse spontaneously and release phage after growth in culture, and they are referred to as **lysogenic**; the phenomenon itself is known as **lysogeny**. Many phages ("temperate" phages) have the ability to establish either lytic or lysogenic infections of their host bacteria. The viral genetic information remains present in the lysogenic bacterial cell, but viral gene expression is very limited; the quiescent viral genome is referred to as the **prophage**.

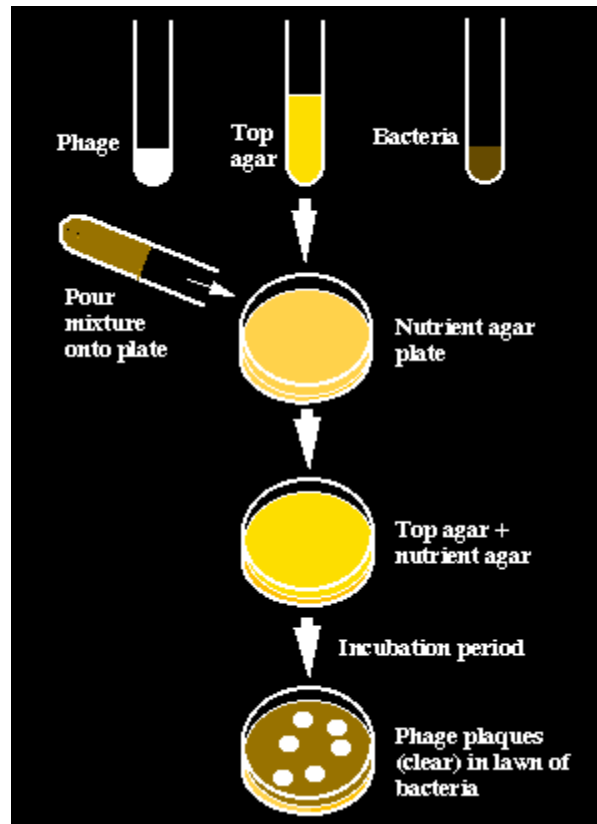
A very well-studied example of a temperate phage is phage lambda, which integrates its DNA genome into the host chromosome using a virally-encoded integrase. Another example is bacteriophage mu (μ , for mutator), which inserts its genome randomly into the host chromosome using the enzyme transposase. These random insertions of the μ prophage cause mutations of the host chromosome (hence the name of this phage); this process, which is mimicked by some mammalian retroviruses, is known as insertional mutagenesis. A third example of a temperate phage is P1. The prophage of this phage is a plasmid, a self-replicating autonomous chromosome within the bacterial cell. Studies on phages of this kind have provided many important and instructive insights into the behaviour of mammalian viruses such as retroviruses -- viruses which can also integrate their genetic information in the host chromosome, and establish a reversibly non-productive interaction with their host cells (in this case, known as a latency).

Analysis of phage

Plaque assay. When a phage infects a layer or lawn of bacterial cells that are growing on a flat surface, a zone of lysis or growth inhibition can occur. This produces a clear zone in the bacterial lawn, known as a plaque. Each plaque originates from a single phage particle.

Plaque assays for bacteriophages are performed by mixing the phage into a layer of bacteria which are spread out as an overlay on the surface of an agar plate. As the plate is incubated, the bacteria grow and they become visible as a turbid layer on the plate. The plaques form clear areas in this lawn. The plaque assay is useful not only because it allows for quantitation of phage, but also because it allows for identification of pure phage strains (since each plaque arises from a single phage). This is very important in the molecular biological applications of phage vectors.

Overview of plaque assay

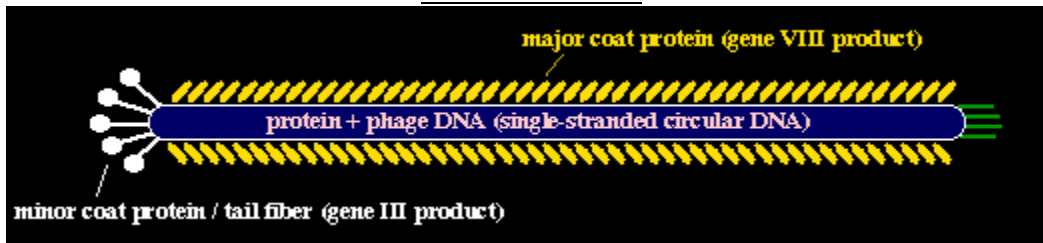


Examples of phages which have applications in molecular biology

M13: A single-stranded filamentous DNA bacteriophage. This phage infects only male bacterial cells, after attachment to the male-specific pilus (F pilus, present in suitable host cells with the genotype *E. coli F*). Upon entering the cell, the phage is stripped of its protein coat, and the single-stranded DNA is converted into a double stranded replicative form (RF). This is followed by DNA replication and assembly of new particles. Roughly 200 phage particles are produced per infected cell, per generation. M13 and the other filamentous DNA phages are unusual in that they are released from the infected cell, without causing the death of the cell. Thus, phage plaques of M13 are seen as areas of reduced growth in the bacterial lawn (i.e., clear zones of bacterial lysis do not occur). Two other unusual features of M13 are (i) stability to extremes of pH and temperature (pH as low as ~pH 3, and temperature up to 55°C) and (ii) its unusual shape and length

(it is rod-shaped, and approx. 6 nm in diameter and 860 nm in length). M13 has historically been used a cloning vehicle, and it has also been widely used for DNA sequencing (this is because the viral particle contains single-stranded DNA, which is an ideal template for DNA sequencing). More current applications of M13 phage include its applications in phage display technology (see below).

Structure of M13

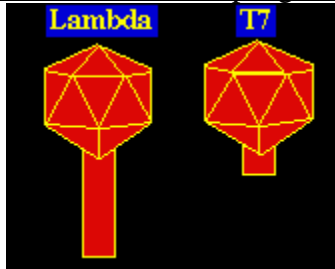


Phagemid: Phagemid vectors such as pBlueScript or many other commercially available vectors contain both phage (M13) and plasmid origins of DNA replication. As a result, they replicate as plasmids in *E. coli*, and they can also be packaged as recombinant M13 phage in the presence of helper phage. An example of such a helper phage is the M13 strain KO7, which contains all of the viral structural genes (absent in the phagemid) as well as a defective origin of DNA replication. This origin of DNA replication is sufficiently active to permit propagation of the KO7 phage, but it is much weaker than the origin contained in phagemid vectors. As a result, infection of phagemid-containing bacterial cells with KO7 helper phage results in the packaging of only the phagemid.

T7 phage: This is one of several double-stranded DNA phages. It is closely related to T3 phage, and infects *E. coli* as well as some strains of *Shigella* and *Pasteurella*. It has an icosahedral head and a very short tail; its overall dimensions are very similar to those of mammalian viruses such as adenovirus. Its genome is approximately 40 kb in length, and it lyses its host cells. T7 phage vectors are presently used both for cDNA cloning and for the generation of surface display libraries (see <http://www.novagen.com>). In addition, the T7 bacteriophage promoter and RNA polymerase are in widespread use as a system for regulatable or high-level gene expression. The T7 RNA polymerase recognizes only T7 promoters. For use in recombinant DNA applications, it is

typically placed under the control of a regulatable promoter such as the *E. coli* lac promoter, and it is then used to drive expression from the T7 promoter.

Structure of T7 and of phage lambda



Lambda Phage: λ phage is perhaps the most well characterized temperate bacteriophage. Infection by lambda can result either in lysis (death of the host cell) or in lysogeny (persistence of the viral prophage in the host cell, with minimal viral gene expression). A small proportion (~0.1% or less) of lysogenic bacteria undergo spontaneous reactivation of the prophage, produce viral particles and die. Lysogenic cultures can also be treated in such a way that most or all of the cells will undergo lysis. This is referred to as induction, and it typically involves the exposure of bacteria to UV light, X-rays or other DNA damaging agents (interestingly, these agents also activate the replication of several mammalian viruses, including HIV-1 and adeno-associated virus).

The regulation of lambda reproduction is very well studied, and it involves a genetic switch that regulates the transition between lysogeny and lysis. In the lysogenic bacterium, only a single phage protein is produced -- the *cl* repressor protein. This binds to two operators on the viral genome and thereby shuts off the transcription of all of the other genes of the phage. When DNA damage occurs in the bacterial cell, the RecA protein is activated, and converted into a protease, which degrades *cl*. This allows for the onset of lytic gene expression.

Lambda phage is an important model system for the latent infection of mammalian cells by retroviruses, and it has also been widely used for cloning purposes. It remains in use for purposes of cDNA cloning and expression, as well as for genomic DNA cloning.

Selected Applications of Phage Technology

"Natural" antibiotics: In the early years following the discovery of bacteriophages, there was considerable interest in using these viruses in bacterial disease prophylaxis. However, early claims of success were overstated. In fact, one commercial phage preparation which appeared in the 1930s ("Enterophagos") was described as being effective against all manner of non-bacterial ailments, including herpes infections and eczema. As a result of these problems, and because of the discovery of antibiotics, research into the use of phage therapies for the treatment of bacterial infections came to a near standstill -- although the therapy did continue to be researched in the Soviet Union.

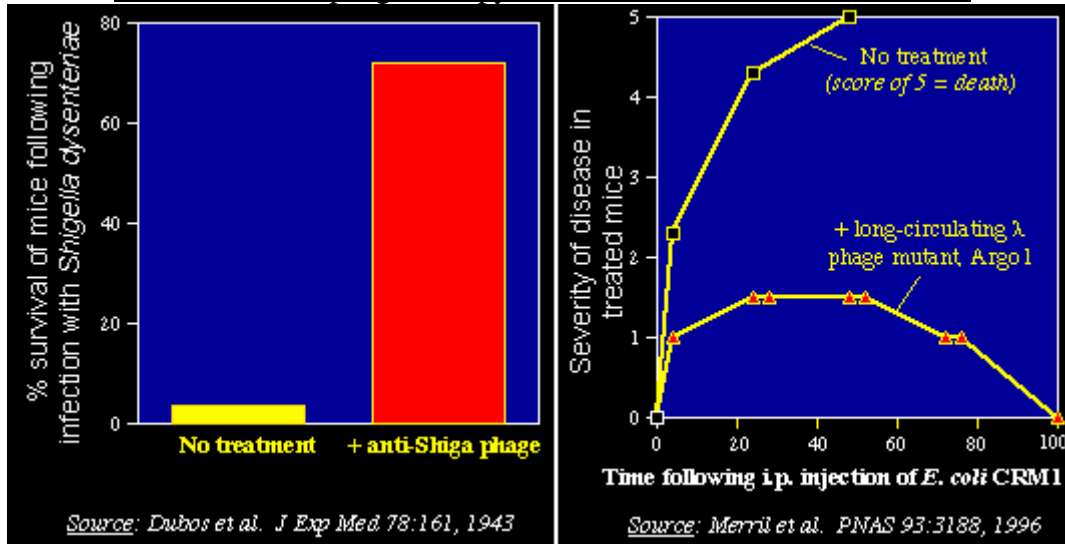
More recently, this issue has become to come to the fore once more, due to the increasing prevalence of antibiotic-resistant bacterial strains. Evaluation of the results from some rigorous experiments in the 1940s reveals that this approach may have promise. For example, a study by René Dubos and colleagues showed that shiga-specific phage preparations could protect mice from the otherwise lethal effects of *Shigella dysenteriae* (next page). These workers also showed that the phage were able to replicate along with the bacteria, and that the phage multiplied *in situ* in the brain of mice inoculated intracerebrally with *Shigella*. Conceptual advantages of phage therapy for bacterial infections includes the following:

1. A single dose of phage should treat an infection, since the phage will replicate themselves.
2. Phage are non-toxic and highly specific for targeted bacterial populations (unlike antibiotics which also kill normal flora).
3. Antibiotic-resistant phage remain sensitive to phage-mediated lysis.

Among the problems associated with phage therapy are the rather narrow specificity of given phages, the possibility of bacterial resistance to phage attack, and the potential for lysogeny when using temperate phage. Another concern relates to the fact that over 90% of administered phage is eliminated from the circulatory system within a very short time frame, thereby limiting the pool of

phage which is available for infecting bacteria. Merrill and coworkers addressed this issue by performing an *in vivo* selection for phage mutants with the ability to persist at high titer in circulation. In this way they isolated long-circulating phage mutants ($T_{1/2}$ *in vivo* of approx. 6 hrs, versus approx. 1 hr for wild-type phage). These phage mutants exhibited a potent anti-bacterial effect when tested in mice that were challenged with a lethal dose of *E. coli* CRM1 (see below).

Effectiveness of phage therapy for treatment of bacterial infections



Molecular Biologic Uses of Bacteriophage

DNA and cDNA cloning

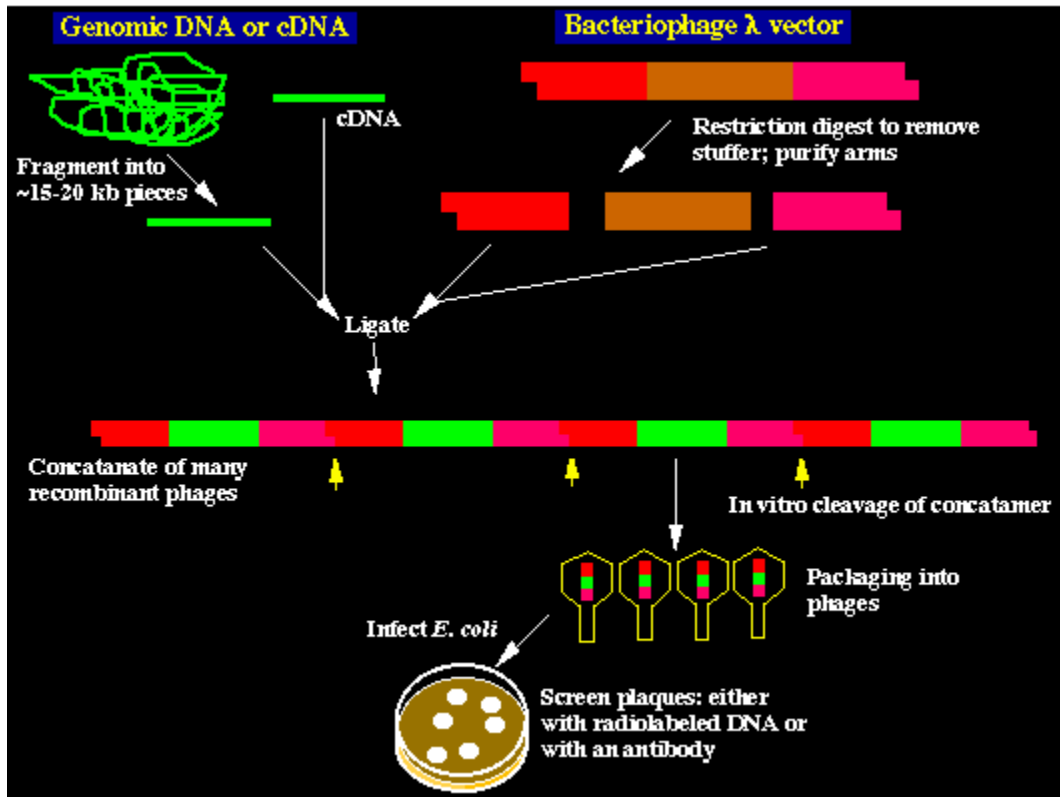
A number of phage vectors are used in DNA and cDNA cloning. Perhaps the most widely used are phage lambda vectors, which can be used both for cloning of mammalian DNA fragments (genomic DNA libraries) and for the cloning and expression of mammalian cDNAs (cDNA libraries). The overall cloning strategy is illustrated on the next page. Briefly, genomic DNA is fragmented into pieces of about 15-20 kb in size and then ligated to the "arms" of a pre-digested lambda phage cloning vector. This results in the production of concatenated molecules which are cleaved and packaged into phage heads using commercially available packaging reactions. The packaged phage particles are used to infect susceptible *E. coli* strains (strains are usually grown in the presence of maltose,

since this induces expression of the bacterial *lamB* gene, which transports maltose into the cell and serves as the receptor for lambda phage). The phage plaques which result from infection of susceptible bacteria can then be screened with radioactive DNA probes or with antibodies, to detect the desired DNA fragment or the protein product of the desired cDNA clone.

Important considerations in the use of phage lambda as a cloning vehicle include the following:

1. The vector systems are of the **gene-replacement** type. That is, the final recombinant clones remain infectious for *E. coli* since the genes which are deleted from the phage are non-essential essential for lytic phage growth. Thus, typical lambda phage vectors are in many ways analogous to certain mammalian virus vectors - such as vaccinia virus or herpesvirus vectors in which a foreign gene has replaced some non-essential viral gene (such as the gene encoding thymidine kinase).
2. The phage's genome cleavage and packaging machinery makes specific nucleolytic cleavages at the cohesive ends between concatemeric genomes (so called **cos sites**). This releases the genome unit-length molecules for packaging. Many mammalian viruses also have specific terminal sequences which are important for genome replication and packaging.
3. The wild-type phage genome (i.e., 38-53 kb or so), since the lambda phage head has a tight constraint on the amount of DNA that it will accommodate. The **packaging size limitation** of lambda phage vectors is one which is common to most mammalian virus vectors also (i.e., recombinant viral genomes must usually be of wild-type length $\pm 5\%$ or so). Two of the few exceptions are the bacteriophage M13 and mammalian rhabdoviruses (such as vesicular stomatitis virus), both of which can accommodate genomes considerably (>10%) larger than wild-type. This is probably because of the rod-like structure of these viruses (bullet-shape in the case of rhabdoviruses); an increase in the length of the rod allows the particles of these viruses to accommodate an extended genome.

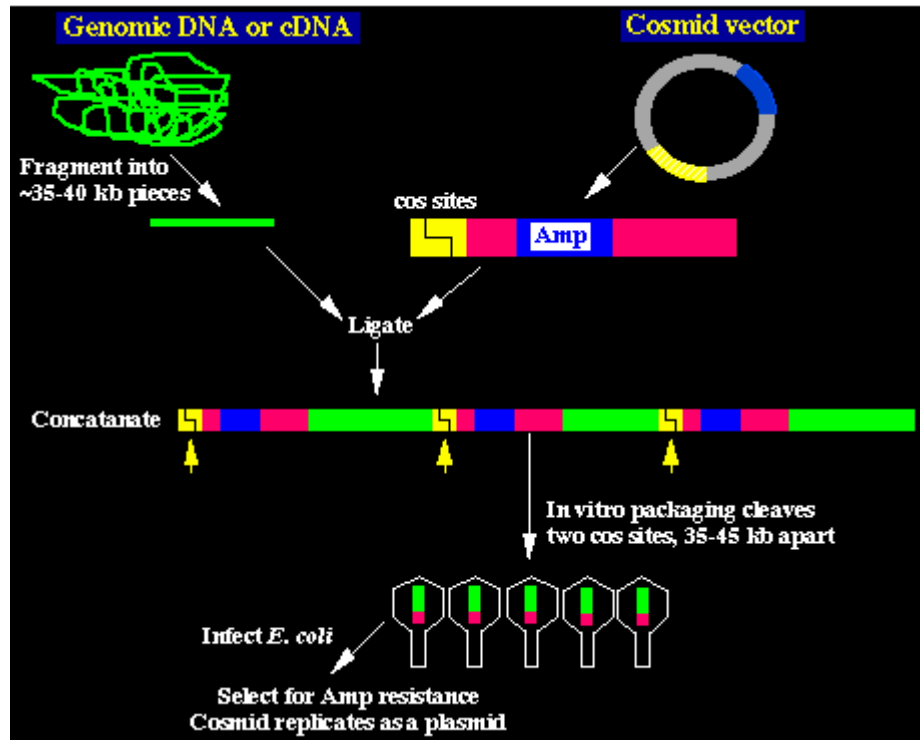
Use of phage lambda as a DNA cloning vehicle



A variation on the idea of using phage lambda as a cloning vehicle is to take advantage of the specificity of the viral cleavage/packaging apparatus, by using plasmid vectors which contain the cohesive termini (cos sites) from the phage. Such vectors are known as **cosmids**. Unlike standard lambda phage vectors, cosmid vectors are not infectious for *E. coli* and do not form plaques. However, because they contain a conventional plasmid origin of DNA replication and selectable marker(s), cosmids can be readily propagated and analysed in *E. coli*. The major advantage of such vectors is their enhanced insert capacity (up to 35-40 kb, since they contain no phage genes other than the cos sites).

The inability of cosmid vectors to undergo autonomous replication in *E. coli* make this vector system an example of a defective virus vector system. Since cosmids rely on exogenously supplied protein extracts for packaging, these vectors can be viewed as being analogous to other **helper-dependent, replication-defective** virus vector systems such as herpesvirus amplicons or certain types of adeno-associated virus vectors.

Cloning strategy for cosmid vectors



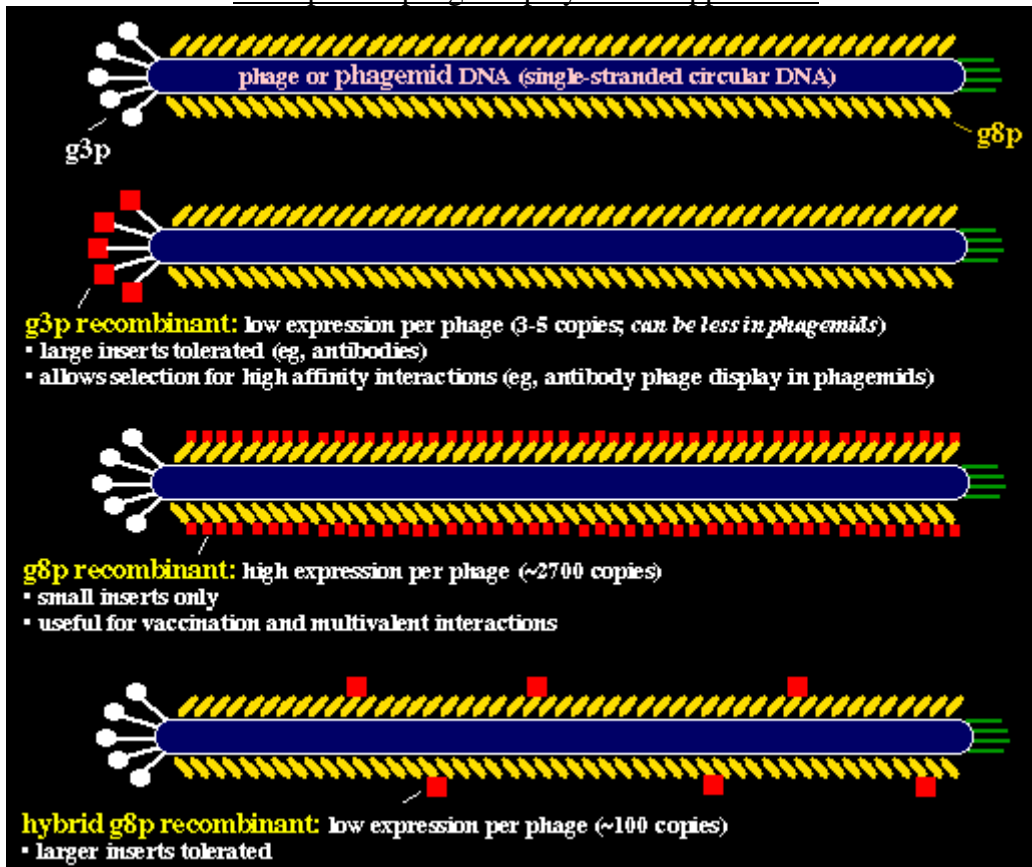
Phage Display Technology

Phage display technology involves the expression of exogenous proteins, peptides or peptide libraries on the surface of bacteriophage. The most commonly used phage for this work is M13 (or other filamentous phage). However, T7-based display systems have also become available commercially and offer a useful alternative approach.

Applications of this technology include the identification of novel peptides with the ability to bind to defined target molecules (e.g., cell surface receptors or enzymes), and the identification of human monoclonal antibodies with defined reactivity against specific targets (in this case, antibody phage display libraries are used). Other examples of the uses of this technology include the use of recombinant bacteriophage displaying antigens from infectious disease agents as candidate vaccines, to confer immune responses against the encoded

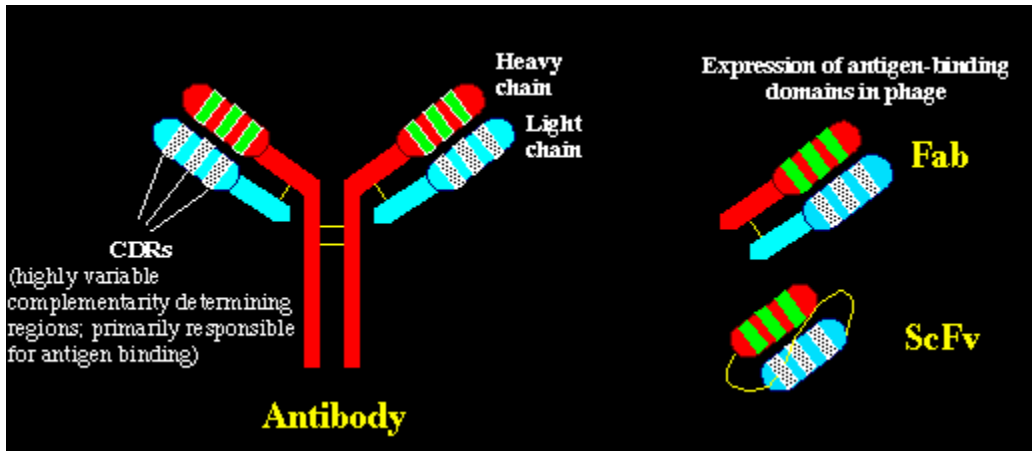
peptides or proteins. Advantages of the technology include its simplicity, ease-of-use, low cost, safety and immunogenicity (for vaccine studies).

Examples of phage display vector approaches



In the case of **antibody display libraries**, phagemid vectors are typically used (since they have a very large insert capacity) and the vector system is generally selected such that there is an average of perhaps 0.1 copies of antibody per phage. Thus, most phage lack any displayed antibody, and almost no phage will display any more than a single antibody molecule.

Antibody molecule, and fragments expressed by phage display



Design of peptide display libraries. The design of random peptide phage display **libraries** typically involves the in-frame insertion of a short random peptide into the M13 minor coat protein, g3p (gene III protein). As a result, the peptide is displayed on the surface of the bacteriophage and the phage remains infectious for *E. coli* (i.e., the functional activity of the g3p remains intact). *Note: in the case of phagemid vectors, the vector is not infectious, since it requires the presence of a helper phage for replication and packaging.*

Typical peptide inserts range from 6 to 15 amino acids in length, and they may be either linear or cysteine-constrained (i.e., linear N₆₋₁₅ or the same insert flanked by two fixed cysteine residues, to allow disulfide formation; C-N₆₋₁₅-C). Linear inserts will assume a three-dimensional configuration (shape) that may be influenced by the coat protein residues immediately flanking the inserted peptide, while disulfide-constrained inserts will assume a more defined shape since they will be contained within an exposed, covalently closed loop on the surface of the phage particle. The maximum possible diversity of different peptide sequences or shapes within a given library is determined by the length of the peptide insert (for example, a random heptapeptide library would contain up to 20⁷ different clones, for a total theoretical diversity of 1.28 x 10⁹). In practical terms, for libraries containing peptide inserts of 8 amino acids or greater, the total complexity of the library is limited not by theoretical constraints, but by practical and methodological considerations which make it hard to derive libraries with a total complexity of much above 10¹¹).

Screening of phage display libraries. Phage display libraries have been used to identify the following: antibody epitopes, protein-protein binding sites, peptide mimics of non-peptide antigens, receptor-binding molecules, protease cleavage sites, novel enzyme inhibitors (*Dennis et al. Nature Biotech. 404:465, 2000*) and tissue- or tumor- targeting peptides (*Arap et al. Science 279:377, 1998*). The latter have been used to re-target viral gene delivery vectors to particular cell or tissue targets of interest, including tumor cells (*Romanczuk et al. Human Gene Ther. 10:2615, 2000; Liu et al. J. Virol. 74:5320, 2000*).

The isolation of novel peptides or proteins (antibodies) with reactivity against defined target molecules is most commonly achieved by iterative cycles of **biopanning**. Biopanning is typically performed by incubating a library of phage-displayed peptides or antibodies with a target molecule of interest, immobilized either on a plastic plate or on paramagnetic beads. The phage is allowed to bind to the immobilized target, after which the unbound phage is washed away and the bound material is eluted. The eluted phage is then re-amplified and several additional cycles of binding and amplification are performed in order to enrich for phage clones which have the ability to bind to the desired target. Ultimately, the clones are sequenced and a consensus binding motif is identified (in the case of peptide display libraries). Typically, this takes from 2-4 cycles of biopanning. Variations on this general methodology include direct biopanning against intact cells of interest (e.g., cells from the respiratory epithelium were used in one study, which aimed at developing peptides that might retarget adenovirus vectors to lung tissue). In other studies, phage libraries have been directly introduced into live animals, in order to select for peptide sequences which confer the ability to home to selected tissues.

Biopanning of phage display libraries

