PHAGE DISPLAY – A TOOL FOR DETECTION AND PREVENTION AGAINST PATHOGENS. A REVIEW

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Abstract: In this article are reviewed the promising uses of phage display in the areas such as microbial pathogens detection of and vaccination. Phage display is a molecular technique by which foreign proteins are expressed at the surface of phage particles. Such phages thereby become vehicles for expression that not only carry within them the nucleotide sequence encoding expressed proteins, but have also the capability to replicate. Recent data acquired from genome sequencing and advances in phage biology research have aided the development of phage-derived bacterial detection and treatment strategies.

Key words: phage display, microbial pathogens, detection

1. Introduction

Bacteriophages (phages) are viruses that infect bacteria. Phages are either virulent (lytic) or temperate and use the host bacterium as a factory for their own replication. Since their independent discovery by Twort and d'Herelle, research on phage has enabled major fundamental and technological advances that have been essential for the emergence of modern molecular biology (MCAULIFFE *et al.,* 2007). In fact, it is difficult to over-emphasize the large impact that phage research has had on molecular biology. For example, phage research was cardinal in defining the central dogma of molecular biology, in the identification of DNA as the genetic material, the definition and mapping of the gene, and the discovery of mRNA as an intermediate for the production of proteins. Furthermore, phages were used, simply and elegantly, to demonstrate the random pre-existing nature of genetic mutations before evolutionary selection, providing clear evidence of Darwin's natural selection theory (CAIRNS *et al.,* 2006). Our understanding of gene regulation has also profited immensely from studies using lambda phage (λ) (PTASHNE, 2004). In molecular biology, the impact of phage-derived technology is clearly visible: transducing phage, restriction enzymes, phage promoter-based expression systems, random mutagenesis (e.g. phage Mu), phage-based cloning vectors and genomic libraries, and phage display techniques, to name but a few. The sheer abundance of phage on Earth (estimated to be 1031 (WOMMACK and COLWELL, 2000)) and the accumulating data from both phage and bacterial genome sequencing projects have highlighted the global significance of phage in bacterial ecosystems and their key role in the adaptive evolution of bacteria.

Sequence analysis indicates that up to 20% of each bacterial genome might consist of phage-related DNA in the form of prophage or phage remnants (CASJENS, 2003). The importance of these phage-related sequences in the evolution of bacteria is most striking in the phenomenon of lysogenic conversion. For example, in *Vibrio cholerae*, the genes encoding cholera toxin are provided by a converting phage, resulting in a virulent strain (WALDOR and FRIEDMAN, 2005). Phage are genetically diverse; approximately half of newly sequenced phage genes have no known homologues (HAMBLY and SUTTLE, 2005), and phage genomes vary in size by at least two orders of magnitude (KUTTER, 2005).

This review aims to provide an insight into how an understanding of phage biology can be exploited to generate novel bacterial detection and vaccination strategies.

2. Phage display principles

In phage display, a heterologous peptide or protein is displayed on the surface of the phage (Fig. 1) through transcriptional fusion with a coat-protein gene (SMITH, 1985), which is accomplished by the incorporation of the nucleotide sequence encoding the protein to be displayed into a phage or phagemid genome as a fusion to a gene encoding a phage coat protein. This fusion ensures that as phage particles are assembled, the protein to be displayed is presented at the surface of the mature phage, while the sequence encoding it is contained within the same phage particle. This physical link between the phenotype and genotype of the expressed protein and the replicative capacity of phage are the structural elements that underpin all phage display technology (Fig. 1) (WILLATS, 2002).

This results in a production a novel phage particle that has a variety of potential uses. The most widely used phage display methods are based on the use of M13 and related filamentous phages of *Escherichia coli* but others, including the *E. coli* phages λ and T7, have also been used (BENHAR, 2001, WILLATS, 2002). Because particulate phages are relatively easy and inexpensive to purify, phage display can also provide a means of purifying a particular protein or antibody. Phage-display libraries can be screened in several ways to isolate displayed peptides or proteins with practical applications. For example, it is possible to isolate displayed peptides that bind target proteins with affinities similar to those of antibodies. They can then be used as therapeutics, which act either as agonists or through the inhibition of receptor–ligand interactions.

The high-affinity, displayed peptides also have the potential to be used for the detection of pathogens and agents posing a biological threat in the environment (PETRENKO and VODYANOY, 2003). A more complete discussion of the potential of phage display is given in several recent reviews (BENHAR, 2001, WILLATS, 2002, WANG and YU, 2004).

The simplest way of achieving the expression of a foreign protein is to simply create a fusion between the nucleotide sequence to be expressed and a coat protein gene within the viral genome (Fig. 2a).

Using this direct approach all the copies of the chosen coat protein become fusion proteins (WINTER *et al.* 1994). This can be advantageous in terms of numbers of

expressed foreign proteins but if the functionality of the chosen coat protein is compromised by the fusion then phage viability may be affected, especially since no wild type versions of the coat protein are retained.
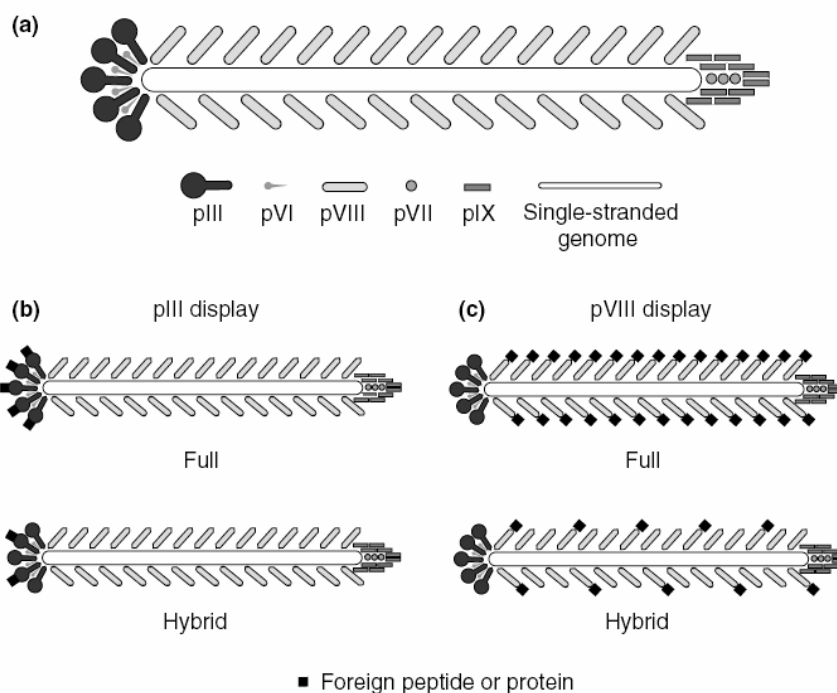
Fig. 1. The principle of phage display. **(a)** Simplified hypothetical bacteriophage with coat proteins pIII, pVI, pVIII, pVII, pIX. **(b)** Forein peptide or protein is dispalyed at the phage surface by the fusion of gene encoding forein protein with phage pIII coat protein coding gene, or **(c)** by the fusion of gene encoding forein protein with phage pVIII coat protein coding gene. The number of copies of forein protein displayed is related to which phage coat protein is chosen as a fusion partner (MELITA *et al.,* 2001).

This can be avoided if hybrid phages are produced in which some versions of a given coat protein are wild type and some are fused to a foreign protein (Fig. 2b and 2c). In some hybrid phage systems, the gene fusion is an additional element of the phage genome so that a wild type copy of the coat protein gene is retained and phage particles express both wild type and fusion proteins (Fig. 2b) (SIDHU, 2001).

Alternatively, hybrid phage may be created using a phagemid-based system and this approach has been widely adopted (Fig. 2c). Sequences encoding fusion proteins are carried by phagemids (plasmids with a phage origin of replication) while the most of the genes required for the formation of phage particles are carried by helper phage that is co-infected together with phagemids into host bacteria (SIDHU, 2001) (Fig. 2c).
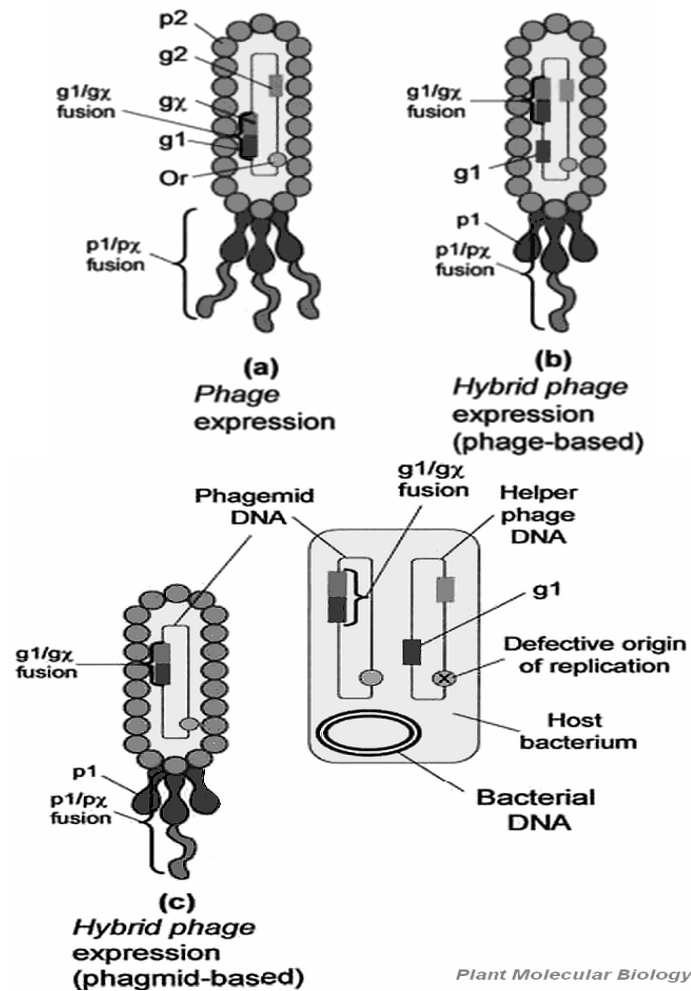
Fig. 2. Strategies for the expression of proteins at the surface of a simplified hypothetical bacteriophage. The simplest format for the expression of a peptide or protein **(a)** is to fuse the gene (gχ) encoding the foreign protein (pχ) to one of the phage coat protein genes (e.g., g1) Hybrid phage **(b)** may be created by incorporating the gene fusion (g1/gχ) as an additional element in the phage genome. With this arrangement, two versions of the phage coat protein chosen as the fusion partner are encoded - one by the native gene (p1) and one by the fusion gene (p1/pχ). As phage particles are assembled both p1 and p1/pχ are incorporated into the phage coat. Phagemid based systems **(c)** are also widely used to construct hybrid phage. However, instead of being present on a single genome, the genes encoding wild type coat protein and fused protein are carried by helper phage and phagemid respectively. Host bacteria contain both phagemid and helper phage DNA and both genomes contribute to the synthesis of hybrid phage particles (WILLATS, 2002).

3. Phages for the detection and typing of bacteria

Many methods of detecting specific bacteria are available, but the low-cost and ready production of large numbers of phage, added to their specificity for a target

bacterial species, makes them ideal for bacterial detection. Although other biomolecules can confer specificity of molecular recognition, they have their own limitations. For example, antibodies are expensive to produce and sensitive to harsh environmental conditions. Another important advantage of phage-based systems is that they usually only detect living bacteria, thereby reducing the number of false positives that arise from the use of approaches such as PCR. Furthermore, dead cells present after processes such as pasteurization or disinfection might be no hazardous but still can be detected by antibodies (PETTY *et al.,* 2007).

Phages bounded to bacteria can be detected by specific labeled antibodies, thereby increasing the sensitivity of detection (WATSON and EVELAND, 1965). For specific typing, different species of phage can be plated out onto a lawn comprising an unidentified bacterial strain, and the presence of clear areas (plaques) where an individual phage particle has grown and lysed the surrounding cells enables identification of the specific bacteria. Recombinant phages are often used in phage-based detection systems (BENHAR, 2001; WILLATS, 2002). The methods that have been used to detect pathogenic bacteria include (i) using phages specifically to deliver reporter genes, e.g. lux (KODIKARA *et al.,* 1991) or green fluorescent protein (FUNATSU *et al.,* 2002), which are expressed after infection of target bacteria; (ii) using phages that have a fluorescent dye covalently attached to the phage coat, and detecting the specific adsorption (HENNES *et al.,* 1995; GOODRIDGE *et al.,* 1999); (iii) the detection of released cellular components, such as adenylate kinase (CORBITT *et al.,* 2000), after specific lysis; and (iv) using phages displaying peptides or antibody fragments that will bind specific bacterial pathogens or toxins. Other means of improving the sensitivity of detection are phage amplification assays. In brief, phage infection of the pathogenic bacterium results in the release of many phage particles that can be detected by a second (non-pathogenic), sensitive bacterial strain. Success with this approach has been seen with the detection of *Mycobacterium tuberculosis* (PETRENKO and VODYANOY, 2003). A more detailed account of these and other techniques is provided elsewhere (KUTTER, 2005) and a selection that emphasizes the range and sensitivity of the technologies used and the diversity of detectable bacterial species is summarized in Table 1 (PETTY *et al.,* 2007).

4. Phages as vaccine delivery vehicles

Phages have been used as potential vaccine delivery vehicles in two different ways: by directly vaccinating with phages carrying vaccine antigens on their surface or by using the phage particle to deliver a DNA vaccine expression cassette that has been incorporated into the phage genome (CLARK and MARCH, 2004).

In phage-display vaccination, phages can be designed to display a specific antigenic peptide or protein on their surface. Alternatively, phages displaying peptide libraries can be screened with a specific antiserum to isolate novel protective antigens or mimetopes – peptides that mimic the secondary structure and antigenic properties of a protective carbohydrate, protein or lipid, despite having a different primary structure (FOLGORI *et al.,* 1994; PHALIPON *et al.,* 1997). The serum of convalescents can also be used to screen phage-display libraries to identify potential vaccines against

a specific disease, without prior knowledge of protective antigens (MEOLA *et al.,* 1995). In a few cases, whole phage particles displaying antigenic proteins have been used as vaccines in animal models (WANG and YU, 2004; IRVING *et al.,* 2001). Specific antibody responses (DI MARZO VERONESE *et al.,* 1994) and protection against an immune challenge have been shown (BASTIEN *et al.,* 1997).

Tab. 1. Applications of the different phage-based antibacterial strategies

Target species	Type of assay	Matrix	Assay time (hours)	Sensitivity	Refs
Listeria monocytogenes	luxAB reporter phage and single-tube	Culture	2	10^2–10^3 cfu/ml	LOESSNER *et al.,* 1996
	luminometer	Spiked salad	22–24	1 cfu/g	
E. coli **0157:H7**	Fluorescently labeled phage and flow	Ground beef	6	2.2 cfu/g	GOODRIDGE *et al.,* 1999
	cytometry	Milk	10	10^1–10^2 cfu/ml	
E. coli **XL1-Blue**	Quorum sensing and bioluminescence	Culture	1.5	10^8 cfu/ml	RIPP *et al.,* 2006
	(luxCDABE)		10.3	1 cfu/ml	
		Spiked lettuce	12.1	10^5 cfu/ml	
		leaves	22.4	10^2 cfu/ml	
E. coli **BL-21**	Biotin-tagged phage and quantum dots nanocomplexes, fluorescence microscopy	Culture	1	10 cfu/ml	EDGAR *et al.,* 2006
	and flow cytometry				
Pseudomonas aeruginosa	Phage amplification	Culture	4	40 cfu/ml	STEWART *et al.,* 1998
E. coli	Phage-mediated release of adenylate kinase	Culture	1	10^4 cfu/ml	BLASCO *et al.,* 1998
Mycobacterium tuberculosis	Phage replication assay	Sputum	2 days	100 cfu/ml	McNERNEY *et al.,* 2004
Microlunatus phosphovorus	Fluorescently labelled phage and epifluorescent microscopy	Activated sludge	1	10^2 cfu/ml	LEE *et al.,* 2006
E. coli **K-12, MG1655**	b-D-galactosidase reporter phage and amperometric detection	Culture	6–8	0.01 cfu/ml	NEUFELD *et al.,* 2003
E. coli **K-12 W3110**	SEnsing of Phage-Triggered Ion Cascade (SEPTIC)	Culture	10 min	1 cfu/ml	DOBOZI-KING *et al.,* 2005
Staphylococcus aureus	SPREETA surface plasmon resonance	Culture	Not stated	10^4 cfu/ml	BALASUBRAMANIAN *et al.,* 2006

Rather than generating a transcriptional fusion to a coat protein, substances could also be artificially conjugated to the surface of phages after growth, which further increases the range of antigens that can be displayed (MOLENAAR *et al.,* 2002). Because phage particles are naturally immunostimulatory (CLARK and MARCH, 2004; KLEINSCHMIDT *et al.,* 1970), an antigen presented on the phage coat would come 'ready conjugated' with a natural adjuvant activity, without the need for separate protein purification and subsequent conjugation to a carrier molecule before immunization. More recently, it has also been shown that unmodified phages can be used to deliver DNA vaccines more efficiently than standard plasmid DNA vaccination (CLARK and MARCH, 2004; MARCH *et al.,* 2004; JEPSON and MARCH, 2004). When compared with standard DNA vaccination, superior antibody responses have been shown in mice (CLARK and MARCH, 2004) and rabbits (MARCH *et al.,* 2004).

5. Conclusion

According to here reviewed information about phage display, this technique/tool has a multipurpose utilization in molecular evolution, analysis of protein/ligand interactions and the generation of antibodies. Taking into account a prevention against pathogenic microorganisms, phage display could be used as a potential vaccine against *Streptococcus agalactiae*, a well-known bovine mastitis pathogen of particular importance because it is highly infectious (spreading from cow to cow by machine milking, unless care is taken to disinfect the teat cups), and causes mainly subclinical infections. As a result, *S. agalactiae* can spread widely within a herd, causing immediate loss due to reduced milk yield, and eventual large losses, when it is finally recognized. Additionally, this pathogen can be transmitted through dairy product to consumers. Therefore, useful vaccine against this bacterium is of great interest. All clinical isolates of *S. agalactiae* express a polysaccharide capsule, with nine capsular serotypes identified so far. The major invasive disease causing serotypes are Ia, Ib, II, and III (SCHUCHAT, 1998). Indeed, it was observed that the protection conferred by capsular polysaccharides is type specific (KASPER *et al.,* 1996). Noncapsular surface antigens are being investigated as potential vaccine candidates or carrier proteins (GRAVEKAMP *et al.,* 1999; STÅLHAMMAR-CARLEMALM *et al.,* 1993). One of these noncapsular antigens, the Sip protein, was highly conserved and produced by every *S. agalactiae* strain examined to date, which included representative isolates of all serotypes (BRODEUR *et al.,* 2000; CHOTÁR *et al.,* 2006). It was also established that Sip-specific antibodies recognized their epitopes at the cell surfaces of different *S. agalactiae* strains (RIOUX *et al.,* 2001). More importantly, the immune response to purified recombinant Sip protein efficiently protected adult mice against experimental infection with *S. agalactiae* strains representing serotypes Ia/c, Ib, II/R, III, V, and VI (BRODEUR *et al.*, 2000). However, the direct use of a peptide presenting by a recombinant phage displaying as a vaccine to examine its protective potential would be simpler and much less expensive than the conventional method of peptide synthesis or recombinant protein preparation and purification for immunization.

The recombinant phage displaying a pathogen specific peptide, in *S. agalactiae* the displaying of mentioned Sip protein or its part, representing an immunogenic mimic of

natural antigen. This could lead to production of antibodies specific to pathogen. Recombinant bacteriophage displaying a disease specific epitope can be use as a vaccine to examine the potential of an epitope of interest to prevent disease. Phage display has also the overwhelming advantage that it is has cheap and easy. It uses standard microbiological techniques that are familiar to all molecular biologists, and its key resources - phage libraries and clones - are replicable and therefore nearly cost-free after their initial construction or selection. It is astonishing to contemplate that within a single tube could be fit a few hundred trillion phage particles displaying billions of different peptide structures - an abundance and diversity from which hundreds of different users with altogether different purposes in mind can select clones of great value. And when that supply has nearly run out, only simple propagation is needed to replenish what is left to satisfy the needs.

Where a safe and efficacious vaccine available, screening and administration of antibiotics would be unnecessary, policies for the prevention of _S. agalactiae_ infections would be simplified and importantly, prevention would be more successful, whereby this approach is applicable to many other pathogenic microorganisms. Additionally, cost-effectiveness analyses invariably demonstrate that vaccination is the most cost-effective of all potential prevention.

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