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9. Phage display: Fundamentals and applications

Sabino Pacheco and Mario Soberón
Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. postal 510-3, Cuernavaca 62250, Morelos, México

Abstract. Phage display technology was first introduced in 1985 by George Smith. It consists in the expression of exogenous polypeptides on the surface of phages particles, which leads to the selection of polypeptides with high affinity and specificity to any target from a phage library. Since its discovery, a large number of peptides and proteins have been displayed in order to study molecular interactions in many fields of biological and medical sciences. Improvement and recent innovations have been development to the phage display technology allowing to expand its usefulness. Here we describe these issues and discuss our experience using phage display to study the interaction of insecticidal Cry toxins of spore-forming *Bacillus thuringiensis* bacteria with its receptors employing different phage libraries that display single chain antibodies (scFv) or random peptides. Finally, we show our last attempts to display the Cry toxins on phages in order to select variants with improved toxicity or different specificity.

Correspondence/Reprint request: Dr. Mario Soberón, Departamento de Microbiología Molecular, Instituto de Biotecnología. Universidad Nacional Autónoma de México. Apdo. postal 510-3, Cuernavaca 62250, Morelos México. E-mail: mario@ibt.unam.mx

Introduction

Phage display is a powerful method for selecting and engineering polypeptides with desired binding specificity. The filamentous phages have been by far the most commonly used system to display different peptides and proteins. The phage display methodology is based in the genetic fusion of foreign proteins to phage coat proteins, then the fused protein is incorporated to the particle during the assembly ending exposed to the solvent conserving its biochemical functionality [1]. Using molecular biology techniques is possible to generate variations in the foreign genes obtaining large and diverse displayed libraries of proteins of interest (POI), which are then selected, usually by binding affinity to particular ligands. The nucleotide sequence encoding the polypeptide selected is deduced from the encapsulated DNA and subsequently characterized [2].

Over the past decade, many display formats have been developed and applied in biological and pharmaceutical research. These technologies use different type of displayed entities, linkage formats and coding strategies. We can divide these systems in two groups: *in vivo* or biological display systems that employ a host cell (such as phage, bacteria, yeast, and mammalian cell displays), and *in vitro* or non-biological systems that use machinery extracted from eukaryotic or prokaryotic cells for synthesis *in vitro* (ribosome, mRNA, or DNA displays and *in vitro* compartmentalization). Regardless of the format, they contain three common components: the POI displayed, its encoding gene and a physic or spatial linker. One of the most used formats is the phage, mainly the filamentous phage, possibly since it was the first method for displaying polypeptides and then many improvement and innovations have been developed in this technology.

This chapter is intended to provide a background of phage display technology and its applications in the study of protein-protein interaction. In particular, we will discuss the use of phage display to study the mode of action of Cry toxins produced by *Bacillus thuringiensis*. These proteins have insecticidal activity and its toxicity is mediated by specific recognition of receptors. Membranal proteins anchored by glicosil-phosphatidil-inositol (GPI), such as Aminopeptidase-N (APN) and Alkaline phosphatase (ALP), and Cadherin-like proteins have been described as potential receptors of Cry toxins [3-5]. Employing different sources of phage libraries displaying antibodies fragments or peptides we have been able to indentify binding epitopes that are involved in the toxin-receptor interaction [6].

1. Fundamentals

1.1. The biology of filamentous bacteriophages

Filamentous bacteriophages (also called phages) are a family of viruses that infect only gram-negatives bacteria via specific absorption to the tip of bacterial structures called pili. Most information about this type of filamentous phages derives from the similar members of the same family of F-specific filamentous (Ff) phage, which are M13, fd and f1. Unlike most bacterial viruses, Ff phage does not kill its host; the particles are produced and secreted from the infected bacteria without cell killing or lysis. The genomes of these phages have been completely sequenced and are 98% homologous and the genes have a similar genetic organization on the phage genome [7]. Therefore, is not rare that their particles are similar in size and shape.

The genome of Ff phage is a circular single-stranded DNA with 6.4 kb in length. The single-stranded DNA (ssDNA) is called (+) strand and it contain eleven genetic products numbered as pI-XI and two intergenic regions (IR) (Figure 1-A). The viral gene products are classified in three groups according their role in the phage biology: the pII, pV and pX are involved in the replication

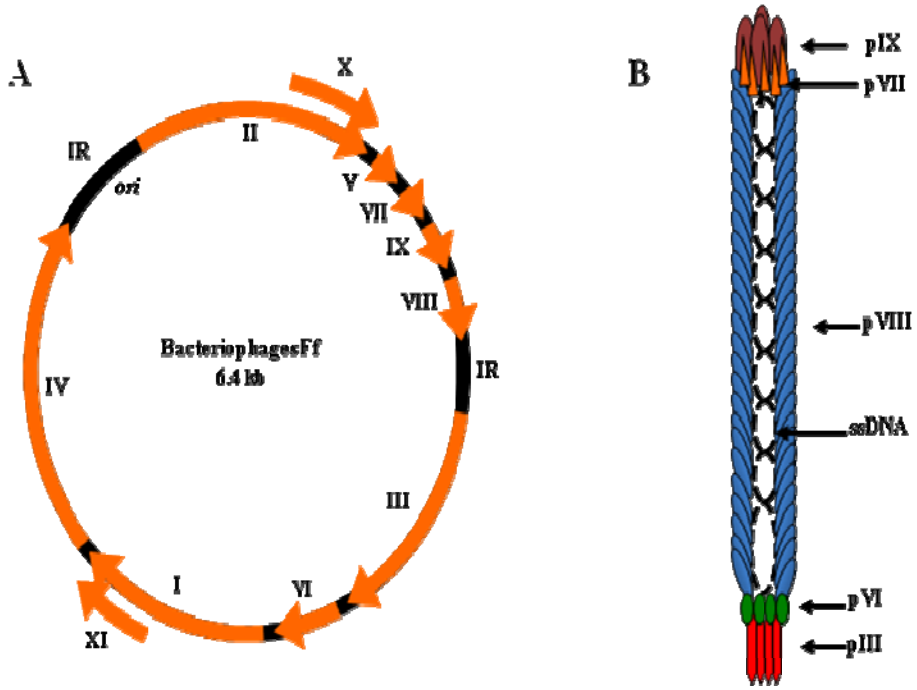

Figure 1. A, Ff phage genome. The genes are numbered of I-XI and their direction of transcription is indicated with arrows. The intergenic regions are labeled as IR and the replication origin is indicated as *ori*. B, Structure of filamentous phage. The structural proteins are indicated with arrows and the encapsulated ssDNA (+).

of the viral DNA; the second group are pIII, pVI, pVII, pVIII and pIX, that are structural proteins which make up the capsid; finally, the pI, pIV and pXI proteins are involved in the viral morphogenesis. The genes X and XI overlap with the genes II and I, respectively. The gene pX is in-frame with pII, therefore is identical in sequence to the C-terminal of pII, while pXI is produced by internal transcription initiation within the gene I. The IR regions are non-coding sequences; the lager IR (between genes II and IV) contains the elements regulating packaging of the viral DNA and its replication [7].

Viral particles have a tubular shape structure surrounding the ssDNA (+) with a fixed diameter of about 6 to 7 nm and a flexible length determined by the size of their genome. The 6.4 kb of Ff phage is encapsided into a 930 nm particle. The viral capsid is composed of five coat proteins (Figure 1-B). The major coat protein is pVIII, a protein of 50 aa that encapsulates the viral genome and forms the cylinder with 2300 copies. The minor coat proteins are located at the ends of the viral particle and are present in few copies. The cap or distal end consists of 3 to 5 copies of pVII and pIX, with a size of 33 and 32 aa, respectively. The tail or proximal end is composed by 3 to 5 copies of pVI and pIII (112 and 406 aa, respectively) [8].

The bacteria are infected by filamentous phage only if it contains a structure called pilus, this is a hair-like appendage on the cell surface involved in adhesion, motility, biofilm formation and DNA transference. There are a wide variety of bacterial pili, Ff phages use as receptor F-pili involved in DNA transference. The infection begins when pIII binds to the tip of a pilus [9]. After phage absorption, pIII and pIV are anchored to the inner membrane binding to a complex composed by TolA, TolQ and TolR proteins, which are co-receptors [10]. They mediate the depolymerization of phage coat proteins and ssDNA (+) translocation into the cytoplasm by an unknown mechanism (Figure 2).

Once the phage ssDNA (+) has entered into the cell, the bacterial machinery synthesizes the (-) strand converting it to double-stranded DNA (dsDNA) by RNA polymerase and extended by DNA polymerase III. The dsDNA is called replicative form (RF), which is template for phage gene expression and further replications. Most viral proteins are transmembrane proteins and are exported to the inner membrane using a signal peptide, with the exception of pII, pV and pX that are cytoplasmic proteins involved in viral DNA replication. The replication is through rolling-circle mechanism, pII nicks the (+) strand in a specific site into the IR (*ori*), then the 3' end is elongated by the host DNA polymerase III using as template the (-) strand and the (+) strand is displaced by Rep helicase. When one round of replication is completed, the (+) strand is nicked in the IR and re-circularized by pII, the newly synthesized ssDNA (+) is converted to RF once again. This

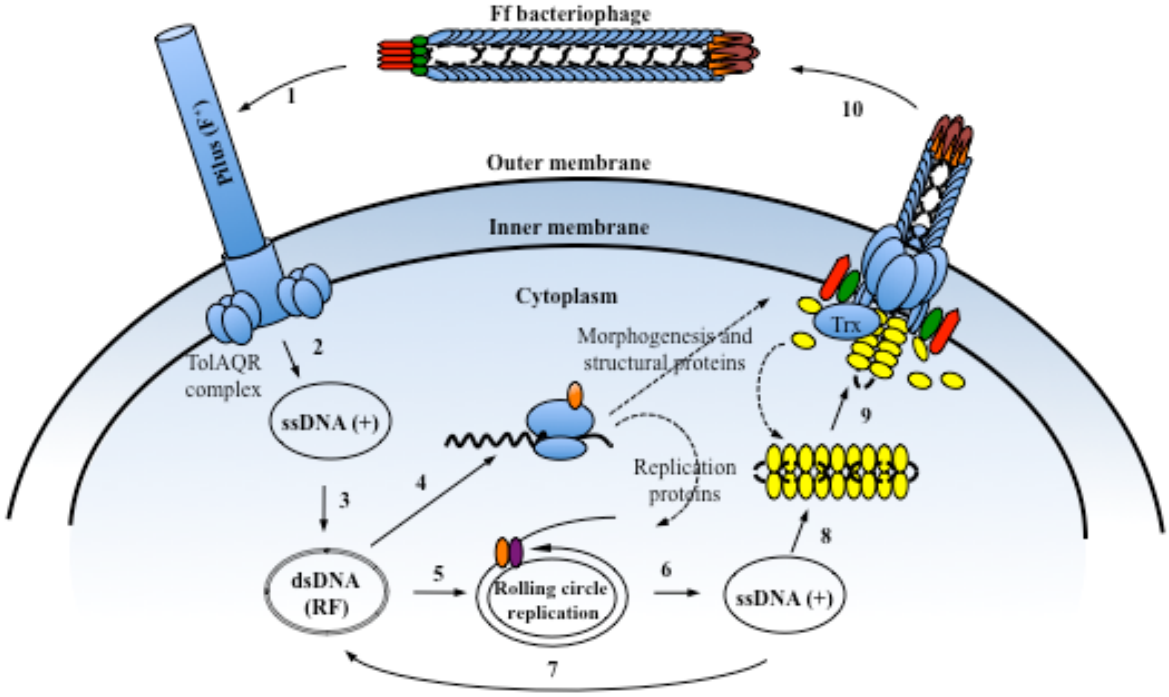

Figure 2. Ff phages infect gram-negative bacteria binding to F-pili (1), then phage particles are absorbed and ssDNA (+) is translocated to the cytoplasm (2) where the (-) strand is synthesized to convert it to RF (3). The RF is the template to gene expression, protein synthesis (4) and DNA replication by rolling-circle mechanism with pII and pX (5). In the early phase of infection the newly synthesized ssDNA (+) are converted to RF (7), but in the late phase forms a complex with pV to avoid this process (8). The pV-ssDNA (+) complex is the substrate to start the phage assembly in the membrane by interaction with assembly site composed by pI, pIV, pXI and Trx (9). The particles are assembled in the inner membrane by incorporation of structural proteins pIII, pVI, pVII, pVIII and pIX, and then secreted to release the mature viruses (10).

cycle is repeated in the early stage infection, but in the late phase, when the pV concentration is high, the ssDNA (+) is trapped avoiding the access to RNA polymerase to convert it to RF. pX, which is identical to 111 aa C-terminal of pII, is a repressor of phage DNA synthesis and limits the RF molecules in the infected cell through an understood mechanism [8].

Unlike most bacterial viruses, the morphogenesis and secretion of filamentous phage occurs in a concerted fashion in the cell membrane where they are assembled in the inner membrane crossing the outer membrane for secretion. The morphogenesis starts with the assembly site formation in the membrane that consists of a multimeric complex composed by pI, pIV and pXI. pI and pXI in the inner membrane connected to the outer membrane by pIV, which forms a homomultimeric channel responsible for phage secretion. The cytoplasmic domain of pI recognizes a DNA packaging sequence signal (PS), localized in the IR protruding from the complex pV-ssDNA (+),

although phage assembly does not utilize redox activity, host-coded cytoplasmic thioredoxins are associated to pI and form part of the complex needed for phage morphogenesis. The assembly starts by incorporation of pVII and pIX at the beginning tip of the particle, these proteins also interact with PS, then the pV dimeric is replaced by pVIII covering the ssDNA (+) and translocating the inner membrane through the pI-pXI complex and then the pIV channel. Finally, pIII and pVI are incorporated to the end of nascent virus and the particle is secreted [8].

1.2. Phage display technology

Phage display is a technology that takes advantage of the bacteriophages biology. In this method a peptide or protein of interest (POI) is displayed on the surface phage particle. This is achieved by cloning in-frame the gene encoding the POI with one of the viral coat proteins, thus during the phage morphogenesis the POI is synthesized fused to the coat protein and assembled on the viral particle. The foreign DNA is cloned to the end terminal of the structural viral protein to be exposed to the solvent. Thus, the displayed molecules often retain its biochemical properties [1]. In addition, using molecular biology techniques, diversity in the gene inserted is generated resulting in a heterogeneous mixture of phages clones, called phage library. In a phage library, each phage clone carries a different foreign DNA and therefore displays a different POI on its surface.

The five coat proteins have been used to display polypeptides on Ff phage [2,11]. However, pIII and pVIII have been most used for displaying purposes. pVIII is used commonly to display short peptides (6 to 8 aa) polyvalently due to its abundance on the capsid phage, larger fragments avoid the viral particle secretion through the pIV channel during the assembly. pIII tolerates larger insertions, foreign proteins are displayed in low copies fusing to complete or truncated pIII versions, although infectivity can be reduced and sometimes eliminated. The POI can be displayed using vectors based in the Ff genome (phage vectors) or in plasmids containing the Ff *ori* called phagemids, which only has the fusion protein gene and all the *cis*-elements for the assembly and replication (IR *ori*) into the host bacteria (Figure 3). Helper phages are used in combination with phagemid vectors. The helper phage is a derivative phage with mutations in the IR ori that reduce its packaging efficiency but still has the whole genome with the three gene groups. Thus, phagemids only carry the coat protein fused to POI, the helper phage provides the proteins required for replication, morphogenesis and assembly. This combination allows displaying larger proteins preserving the infectivity (pIII display) or assembly (pVIII display) of the filamentous phages [2,11].

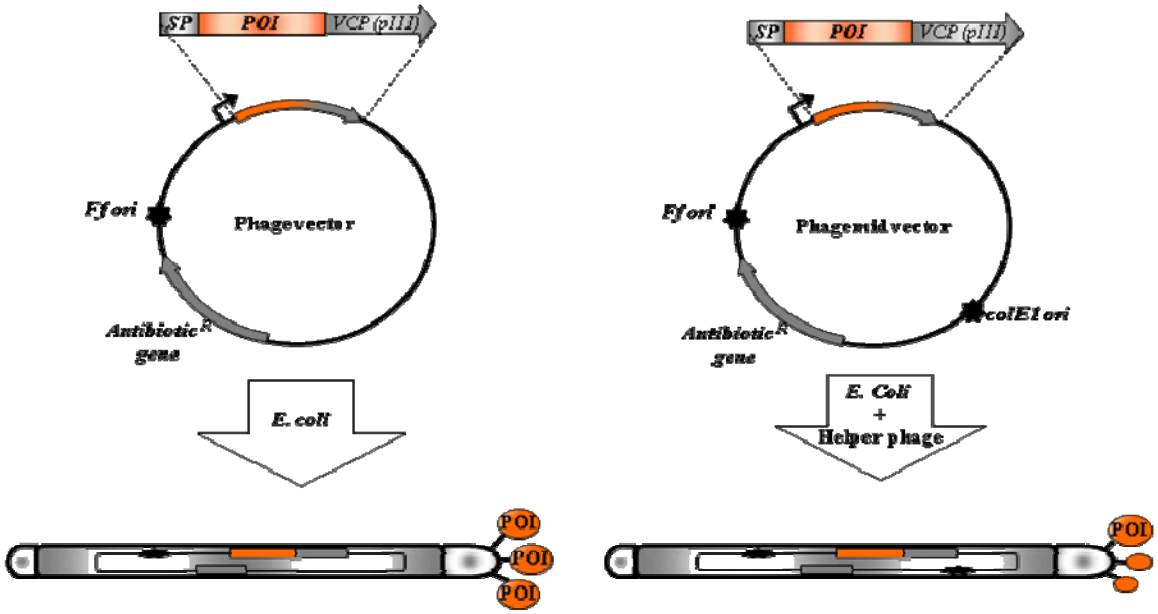

Figure 3. Strategy to display polypeptides on filamentous phage using phage or phagemid vectors. POI is cloned in-frame to the viral coat protein (VCP), pIII in this example. The fusion includes a sequence signal peptide (SP) to translocate the protein through the inner membrane for assembly in the periplasm of the *E. coli* host. Phagemid vectors require a helper phage that supplies the viral proteins including a non-fused pIII. Thus, the filamentous phage assembled carrying a combination of fused and wild-type pIII.

1.3. Biopanning

In general, the starting material for the selection procedure in phage display is a collection of polypeptides variants called library. A large variety of techniques can be exploited to introduce diversity into the gene encoding the POI [2]. The most common approach is oligonucleotide-directed mutagenesis using the polymerase chain reaction (PCR). However, other techniques for obtaining diversity are through DNA-shuffling, error prone PCR or using mutator *E. coli* strains. One of the most used types of libraries in phage display is a collection of antibodies genes, where the variability arises from the broad natural diversity of immunity system [11].

The process to select specific clones against a target desired from a phage library is referred as biopanning (Figure 4). In this procedure the displayed polypeptide is bound to a target tethered to a solid support, then only the retained phages are eluted after washing non-specific and unbounded phages. The selection is based principally on the affinity of polypeptides to the target. The eluted phages displaying polypeptides with higher affinities are used for bacteria re-infection in order to amplify the clones, which are subject to next

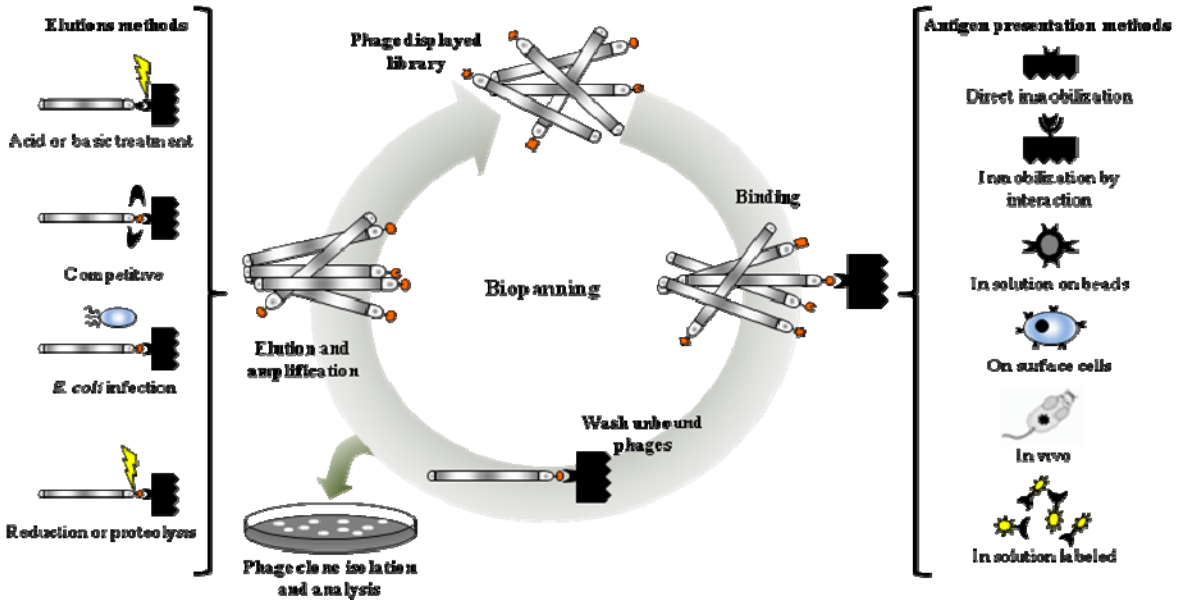

Figure 4. Biopanning procedure involves four principal steps for phage selection. 1. The target is presented to the phage-displayed library allowing the binding (usually the target is immobilized directly to a solid support, such as immunetube, microplates, sensorchips), this step is referred as panning. .2 After incubation, unbound and non-specific phages are washed. 3. The specific phages containing binders to the target can be released by elution methods (usually acid or basic elution following by a neutralization step). 4. The eluted phages are used for *E. coli* re-infection in order to amplify the phages. Due to residual non-specific phages not eliminated by washing, more than one selection round is needed to permit enrichment of best affinity clones.

selection rounds, usually after 3 to 5 rounds of selection are enough to obtain a pool of enriched phages with specific binding and are characterized by traditional biochemical techniques such as ELISA [2,11].

In general, a successful selection implies that the initial library contains a large diversity of displayed polypeptides to increase the chance to select appropriates clones. Selection parameters often can be manipulated in order to enhance the efficiency of phages selection with high or low affinities, such as the antigen presentation and elution conditions [2,11]. Others determinant parameters are intrinsic of the system as feasibility and valence display of the POI on the phages.

1.4. Modifications to improve filamentous phage display

Although many proteins with different size and folding have been displayed successfully on filamentous phage, there are proteins that fail or are poorly displayed. These problems might be consequence of proteolysis, aggregation, inefficient folding of disulfide-containing proteins, toxic effect

in the host cell or incompatibility of signal sequence for its translocation. Therefore, attempts to improve the POI display have been developed.

The co-expression of chaperones, such as Skp or FkpA, in periplasm during the bacteria infection improves the efficient folding of fused proteins and in consequence the display of functional proteins [12,13]. However, the folding of disulfide-containing proteins is a problem that can be overcome by using specialized strains that allow disulfide bound formation. Proteins with an inefficient translocation into the periplasm are poorly displayed. This has been improved by modifications in the translocation signal sequences. There are three pathways for protein translocation through the cytoplasmic membrane of gram-negative bacteria, each pathway recognize specific signal peptides that determine translocation of proteins in different fold-state [14]. Sec and SRP (signal recognition particle) pathways translocate unfolded polypeptides through the translocon Sec. Sec-dependent translocation exports polypeptides post-translationally, while in the SRP pathway is co-translational. The twin-arginine translocation (Tat) pathway translocates folded proteins post-translationally. The efficient translocation of POI through these pathways determines the incorporation to the particle during the assembly for its display level [15-17].

Other intrinsic problem of filamentous phage display system, where the phagemid vectors are used, relies in the requirement of helper phage, such as M13KO7 or VCSM13. Helper phage supplies wild-type versions of the coat proteins required for re-infection of recombinant phage for its amplification. Theoretically, the recombinant phages contain wild-type coat proteins derived from the phage and fused to POI derived from the phagemid. However, in reality the majority of the proteins are wild-type, this implies that a large portion of phages particles do not display POI. To improve display levels using phagemids system, different helper phages have been engineered in such a way that the majority of pIII is fused pIII-POI. These helper phages carry deletions or mutations in the pIII gene that avoid its expression into a specific host strain allowing higher display levels of fused protein derived exclusively of the phagemid [18-22]. *E. coli* strains have been developed with the same function eliminating the need to use helper phage, these cell-lines harbor plasmids that express the phage packaging proteins which assemble phage particles as efficiently as helper phage [23].

During the biopanning process the enrichment for phage displaying high affinity clones over non-specific binders is one of the most difficult tasks. Recent innovations in phage display have been developed to overcome this problem. The selective infective phage (SIP) is a technology that consists in the replacement of the N-terminal domains of pIII by the gene encoding POI, leading to the generation of non-infective particles [24]. The missing

N-terminal domains, necessary for infection, are complemented within adapter molecule consisting of the target covalently linked to these N-terminal domains. Then, the infection is restored when non-infective phages display polypeptides that bind to the target providing the missing N-terminal domains of pIII necessary for infectivity. SIP is a method that avoids the need for physical separation of specific and non-specific binders allowing an efficient and rapid procedure for selection of molecular interactions [24].

1.5. Alternatives phages

The morphogenesis of filamentous phage is performed in the periplasm space; therefore, the structural proteins have to cross through the *E. coli* inner membrane [8]. This characteristic has limitations for filamentous phage displaying proteins whose proprieties avoid its correct translocation. Other limitations derive from the chemical environment of the space periplasmic, which may affects its folding and stability. For overcoming this problem some lytic phages have been used such as T7, λ, T4and P4. These phages have in common that morphogenesis occurs in the cellular cytoplasm; therefore, the coat proteins are folded and assembled into the cytoplasm avoiding secretion through the bacterial membrane. The structure of these phages consists of a head covering its lineal dsDNA genome and a tail involved in the bacterial infection.

T7 phage has an icosahedral head composed of 415 copies of protein 10 (p10), arranged as 60 hexamers on the faces of the shell and 11 pentamers at the vertices. The head is attached to the tail composed by a connector (p8), a short conical tail (p11 and p12) and 6 tail fibers (p17). The viral head normally has two proteins: 10A (344 aa) and 10B (397 aa). 10B is produced by a translational frame shift at the amino acid 341 of 10A, and presents about 10% of the coat proteins. However, functional particles can be composed entirely either 10A or 10B, or various rates of each one. Thus, p10B has been used for phage display since it tolerates extensions that can be incorporated to the capsid. T7Select® is a commercial system for display POI fused to p10B [25]. Three different vectors are used for T7 display: T7Select415 for display peptides of 50 aa in high-copy number (415 per phage), T7Select10 for display peptides or longer proteins in mid-copy number (5-15 per phage) and T7Select1 for display proteins of 1200 aa in low copy number (0.1-1 per phage). A strong promoter, as in T7 wild type, controls the gene 10B expression in T7Select415 vector, therefore the POI is produced in large amount during the infection and the phages are composed entirely by p10B fused, displaying 415 copies on its surface. This polyvalent system is ideal for mapping epitopes or obtaining peptides with low affinity

to their targets. T7Select10 and T7Select1 vectors are suitable for stronger affinity to their target since their promoter have mutations to reduce the p10B production and the host bacteria carry a plasmid that supplies extra p10A allowing that fused p10B are displayed in low-copies number [25].

The λ phage genome is packaged into pre-head that are matured by proteins remotion and proteolytic process, then trimers of pD are incorporated on the surface of the capsid formed by hexa- and pentamers of pE. Finally, when the viral genome is internalized into the head, FII protein is added to the distal part where the tail will be assembled. The tail is composed by rings of pV hexameric, forming a tubular structure flexible through which the DNA is injected to the host bacteria during the infection. pD and pV have been used for λ phage display. pD is a small protein (11 kDa) essential for the phage morphogenesis due to its effect stabilizing, however when the genome is <82% in size, particles can be assembled without pD. POI fused to the ends C- and N-terminal of pD can be assembled in functional phages [26,27]. pV contains two folding domain, the smaller C-terminal domain is exposed to the solvent. λfoo is a vector that allows the expression of POI fused to the end C-terminal of a pV truncated version [28].

T4 is a lytic bacteriophage with an icosahedral capsid formed by three major proteins: p20, p23 and p24. In addition, there are two accessory proteins: SOC (Small Outer Capsid) and HOC (Highly Outer Capsid), that are incorporated to the viral surface after assembly. SOC and HOC are proteins suitable for phage display since they are not essential for assembly and allow the display of two different POI on the same particle. The HOC protein tolerates fusions of 183 aa, peptides but larger proteins affect the affinity of HOC to the capsid affecting phage assembly. Due to its size and abundance SOC is less tolerant for display purposes (960 copies of 9 kDa, while HOC are 160 copies of 40 kDa). A second T4 phage display method was described, in which the POI is fused to the minor T4 fibrous protein "fibritin" encoded by gene *wac* (whisker's antigen control) and displayed on the phage tail [29-32].

P4 phage has a protein (Psu) as a decoration component on its capsid. Psu is non-essential for assembly but enhances the phage stability by binding to its surface. Lindvist and Naderi engineered a P4 phage in which Psu is fused to 10 aa of the human p62c-myc and successful incorporated on the phage head [33]. However, larger proteins fused to Psu have not been studied.

1.6. Others display systems

Since George Smith first showed the display of peptides using phages, other display systems have been developed (Figure 5). Such systems include

in vivo (mammalian cells, yeast and bacteria display) and *in vitro* (ribosome, RNA and DNA display) technologies. These systems, as in phage display, share four essential features; they preserve the link between the POI displayed and its coding DNA sequence, the creation of diversity through libraries, the clone selection by binding against a desired antigen and amplification of selected clones.

Cell-based display or *in vivo* systems are essentially identical to the expression-cloning approach; the POI is cloned in vectors and transformed for its cytoplasmic/periplasmic (intracellular display) or membranal (extracellular display) expression [34-36]. In both cases, the host cell is used to establish the modular link between the encoding DNA and displayed POI. The cellular requirement is a common factor in these systems (including phage display), therefore have the advantage that the polypeptide synthesis is performed by biological machinery and the folding is closest to the native conformation. Nevertheless, the transformation step limits the display efficiency in the system.

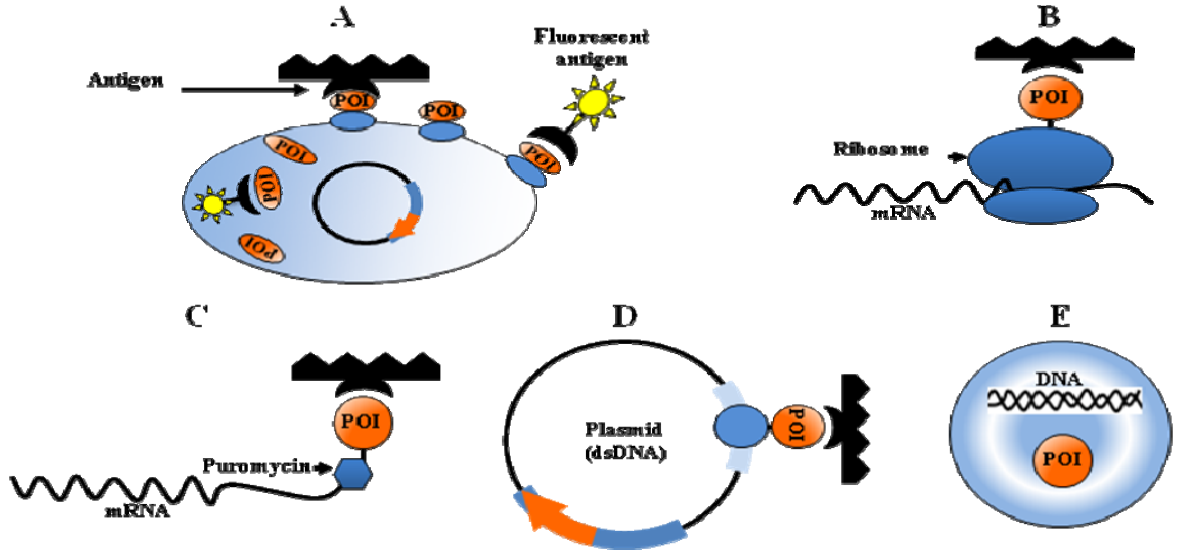

Figure 5. A, Cell display system; POI is displayed by intracellular or membrane expression; therefore the selection is performed by traditional methods with fixed antigen or by fluorescence-activated cell sorting (FACS) using fluorescent ligand. B, Ribosome display system; the mRNA is translated *in vitro* and the nascent polypeptide remains bound to the complex composed by mRNA, ribosome and POI in presence of magnesium ion. C, mRNA display system; puromycin is added to 3' end of mRNA by the ribosome during the translation linking covalently to the POI synthesized. D, DNA display is based in the POI fusion to a DNA-binding protein, which binds to specific DNA plasmid sequence. E, *In vitro* compartmentalization allows the link between POI and its encoding gene by encapsulating into water-in-oil emulsions.

In vitro display systems avoid the need for transformation leading to greater diversity (size over 10^{13}) and an increased probability of higher affinity hits. The transcription and translation steps are performed *in vitro* with machinery extracted from prokaryotic or eukaryotic cells [37]. Ribosome display uses lysates from cells for *in vitro* translation of the mRNA, the nascent polypeptide reminds in a complex (ribosome, mRNA and polypeptide), which can be disrupted by EDTA, the mRNA released is amplified by RT-PCR step [38]. mRNA display (also called in vitro virus) is similar to ribosome display, except that during the translation step the polypeptide is covalently coupled to its mRNA via puromycin [39]. Puromycin mimics amino-acyl tRNA and is bound covalently to the nascent polypeptide as result of the peptidyl transferase activity of the ribosome. However, both system have the disadvantage that they are very sensitive to RNAase degradation, DNA display (also called *CIS* display) is less sensitive because uses DNA as template. In this system the POI is fused to a DNA-binding protein that binds to the plasmid carrying on the encoding DNA [40]. *In vitro* compartmentalization (IVC) is a display system used extensively in the selection of enzymatic activities. Although there is not a physic link between genotype and phenotype, the linkage occurs by encapsulating into water-in-oil emulsions as an aqueous core for in vitro translation reaction [41]. However, the efficiency may be reduced by incomplete separation of droplets or by fusion of the compartments.

2. Applications

The earliest application of phage display was the selection of peptides that bind cell-surface receptors. Currently, this has been expanded and include target as enzymes, intracellular signaling proteins, ion channels, cell surface architectures, non-proteins targets (i.e. DNA, carbohydrates and special surface materials), and protein engineering. Several reviews and books have been published about innovations and evolution of these issues, hence here will not be discussed in detail. We will focus the discussion with examples from the work carried out in our lab using phage display for studying the interaction of the insecticidal Cry toxins from *B. thuringiensis* and its receptors in the insect gut.

2.1. Epitope mapping by displaying peptides

The surface in the ligand that makes contact with the receptor is called epitope, in proteins comprising a few adjacent residues in the primary structure (linear or continuous epitope) or distant but close in the folded

conformation (conformational or discontinuous epitope). In both cases, many epitopes are "conformation-dependent" to adopt a binding conformation, which just few residues are physically contacted and the rest form the geometrical shape. Several methods and technologies (i.e. protein fragments expression, synthetic peptides, phage display or structural analysis) have been developed to map epitopes, this is essential in diagnostics, immunotherapy and vaccine development. The screening of phage libraries displaying random peptides encoding by synthetic oligonucleotide can help to indentify peptides that match the primary sequence of linear epitopes [2]. Frequently, the selected peptides may not have similarity to any linear sequence of the antigen but represent a conformation dependent interaction that mimic discontinuous epitopes and are referred as mimotopes.

Several commercial libraries displaying random peptides are available. In the case of Cry toxins we used one from New England BioLabs Inc. (Ph.D.-12 Phage Display Peptide Library Kit). This library is based on a combinatorial library of random 12-mers fused to the coat protein pIII of the M13 phage. The library was panned using as target the Dipteral-specific Cry11Aa toxin or brush border membrane vesicles (BBMV) from midgut epithelial cells of *Aedes aegypti*. We isolated phages that bind to Cry11Aa toxin (P5-tox) or to *A. aegypti* BBMV (P1-BBMV) [42-44]. The binding of both phages was competed by a synthetic peptide corresponding to a putative loop α-8 of Cry11Aa protein, suggesting that phage P5-tox binds the toxin by interacting with this loop, while P1-BBMV interacts with the receptor using an epitope similar to this region. However, none of peptides sequence selected from phages library match with Cry11Aa toxin or the receptor ALP from *A. aegypti*, this is a clear example of mimotopes isolation.

2.2. Mimics receptors with antibodies

Phage display has been used widely to display antibodies fragments against diverse set of target antigen, which can be useful in multiple applications [11]. One of the major advantages of antibody phage display, compared with standard hybridoma technology, is that generation of specific antibodies against the target can be performed within few weeks. In addition, it lacks of necessity the immunizations protocols and bypass problems with non-immunogenic or toxics antigens [45].

Due the complex structure of antibodies and the limited size to display proteins in phages, only the antigen recognition region of antibodies is displayed. This is determined mainly by complementary determinant recognition loop region (CDR) in the variable light (VL) and heavy (VH) chains. Antibodies phage libraries are based mainly displaying fragments

antibodies in two formats: Fab and single chain antibodies linked by a flexible peptide (scFv) [11]. Also, single VH or VL chains and camel VHH domains have also been used successfully [46-48]. The genomic information encoding for antibodies variable domains is usually derived form B cells either naïve from non-immunized or immunized individuals. Naïve libraries have the advantage that they can be used for an unlimited range of antigens, while the repertoire from immunization is restricted to generating antibodies against an antigen during the immunogenic response. Both libraries contain natural CDRs, however synthetic antibodies phage libraries can be constructed introducing diversity in the CDRs varying loop lengths using PCR and randomized oligonucleotide primers [11]. In order to generate highly diverse repertoire of either synthetic or naïve antibodies fragments, some libraries have been constructed by *in vivo* recombination or combinatorial infection [49]. Thus, the low repertoire imposed by the transformation step being avoided. Antibodies phage display has also been used to select intrabodies in scFv format, which are directed against intracellular target that allow visualizing and modifying of its function [50].

In our lab, synthetic human and immune rabbit scFv phage libraries were used to characterize the interaction between Lepidoptera-specific Cry1Ab toxin and its receptors Cadherin and APN from the insect *Manduca sexta*. The Nissim synthetic antibody phage library, which contains a diverse repertoire of *in vitro* rearranged human VH genes and randomized VH-CDR3 of 4 to 12 aa in length [51], were panned using Cry1Ab protein as target and selected the scFv73 antibody, which compete the binding of Cry1Ab toxin to the Cadherin receptor. Sequence analysis of the CDR3 region led to the identification of a region in Cadherin ($_{869}$HITDTNNK$_{876}$) as binding epitope. The scFv73 antibody also allows map the cognate binding region in the Cry1Ab toxin using synthetic peptides corresponding to three loops of the toxin. Competence analysis shows that the binding of scFv73 with Cry1Ab toxin only is competed by the loop-2 peptide. These results indicate that the Cry1Ab domain II loop-2 region interacts with the Cadherin amino acid region $_{869}$HITDTNNK$_{876}$ region. In addition, scFv73 mimic the Cadherin function; the interaction toxin-receptor allows the Cry1Ab toxin oligomerization, when scFv73 is used instead of Cadherin receptor the same process is triggered. In other hand, we constructed an immune scFv phage library by immunization of a rabbit host with Cry1Ab toxin. After two rounds of competitive panning using Cry1Ab as target and one panning round with synthetic loop-3 peptide as target, we isolated an antibody anti-domain II loop-3 (scFvL3-3). This antibody, as scFv73, competed the binding of Cry1Ab toxin to Cadherin receptor and trigger the oligomerization process. At same time, an antibody anti-domain III β-22 region (scFvM22) was

selected and inhibited the interaction with the APN receptor, but did not promote the formation of an oligomeric structure. Finally, this antibodies set shows that anti-domain II scFv recognize preferentially to the monomeric toxin than the oligomeric, while anti-domain III binds to both structures. These data indicate conformational changes in the Cry1Ab toxin during the oligomarization that allow a binding transition with Cadherin and APN receptors [52-56].

2.3. Directed molecular evolution

All organisms are believed to have evolved by consecutive rounds of diversification, selection and amplification. The diversification process, at the molecular level, is carry out by introducing mutations, such as point mutations, deletions, or recombination, into genes encoding proteins. The resulting proteins confer a phenotype that allows adapting to a selective pressure and fixing the specific mutations that will be inherited to next generation. The success of the process in living cells is due separate their own genes from external environmental and other organism by membranes, thus the genetic information is replicated on basis of their phenotype. Hence, the evolution is based on linkage between genotype and phenotype.

Advances in molecular biology have allowed to mimic this process at the molecular level, such is the case of the display systems for directed evolution of proteins to increase affinities, or stability and design new binders, or activities. The alterations of the properties of proteins are of great interest, as these molecules are useful for industrial use, medical application, and also understanding the biophysical properties of proteins. For this purpose, phage display typically is used to select for binding interaction, such as antibodies or binding proteins using others scaffolds [11,57].

Directed molecular evolution using phage display seems particularly interesting for Cry toxins since for many insect pests there are no Cry toxins available for their control. Also, a major threat for the use of *B. thuringiensis* toxins in transgenic plants is the appearance of insect resistance. It has been demonstrated that a single nucleotide change in the Lepidoptera insect *Heliothis virescens* Cadherin receptor gene produces an amino acid change that abolish Cry1A toxins binding [58]. Therefore, the development of an efficient method that allows genetic evolution of Cry toxins to kill novel targets or to recover toxicity to resistant insects is absolutely necessary. Recently, T7 display system was used successfully to select Cry1Aa toxin by panning variants in the domain II loop-2 region that bound the Lepidoptera insect *Bombix mori* Cadherin with higher affinity [59]. This result shows that it is possible to select novel Cry toxins with desirable binding properties from

a pool of variants. In our lab we display successfully Cry1Ac toxin on T7 phages [60], however the libraries construction with high variability is an intrinsic problem due the need of *in vitro* packaging system for DNA recombinant. Currently, we are improving the Cry toxins display on filamentous M13 phages in order to overcoming this inconvenient.

3. Conclusions

Phage display is a powerful technique that has been fundamental for the study of protein interactions, epitope mapping and protein evolution. Also, it has been important for developing new antibody molecules for diagnostic and therapeutic procedures. In the case of the insecticidal Cry proteins produced by *Bacillus thuringiensis*, phage display methodology has been useful in determining the identity and localization of one receptor molecule in mosquitoes and for defining the sequential participation of two receptor molecules leading to pore formation in Lepidoptera insects. We believe that phage display will also be useful for the *in vitro* evolution of Cry toxins for selecting toxins with new insect specificities and for selecting toxin mutants that could kill resistant insects.

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