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Title:

Tools from viruses: bacteriophage successes and beyond

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Summary

Viruses are ubiquitous and can infect any of the three existing cellular lineages (Archaea, Bacteria and Eukarya). Despite the persisting negative public perception of these entities, scientists learnt how to domesticate some of them. The study of molecular mechanisms essential to the completion of viral cycles has greatly contributed to deciphering fundamental processes in biology. Nowadays, viruses have entered the biotechnological era and numerous applications have already been developed. Viral-derived tools are used to manipulate genetic information, detect, diagnose, control and cure infectious diseases, or even design new structural assemblies. With the recent advances in the field of metagenomics, an overwhelming amount of information on novel viruses has become available. As current tools have been historically developed from a limited number of viruses, the potential of discoveries from new archaeal, bacterial and eukaryotic viruses may be limited only by our understanding of the multiple facets of viral cycles.

1°) Early applications

Amongst the earliest applications of viruses, their use as live or attenuated particles to develop vaccines during the 19th century was very important as it represented the beginning of taming viruses for our purposes, but is beyond the scope of this review. Beside this major contribution to human health, exploitation of viruses ramped up with the discovery of viruses infecting bacteria named bacteriophages. One of the earliest applications of these bacterial viruses was proposed by F. d'Herelle, who demonstrated almost 100 years ago that bacteriophages were able to cure human bacterial infections (d'Herelle, 1917). This therapeutic approach, bacteriophage therapy, rapidly developed globally until the industrial production of antibiotics was launched in the 1940's (Summers, 2001). Around the same time, Delbrück and colleagues chose bacteriophages as model organisms to understand the genetic basis of virus-host interactions (Luria and Delbruck, 1943). Soon after, the larger scientific community adopted these viruses and their hosts, and studies performed during the following 40 years yielded a tremendous amount of literature (Figure 1), allowing major discoveries notably in both DNA and RNA processes shared by living organisms. Altogether, these findings led to the development of the first molecular tools and ultimately to the advent of a new discipline, now called molecular biology (Summers, 1999).

It is remarkable that such a vast array of discoveries were founded on studies performed on a very limited number of bacteriophages (T4, T7, P1, Mu, PhiX174, Lambda and few others but probably less than 20). For example, to our knowledge, the Lambda bacteriophage, one of the best studied temperate bacteriophages, was once isolated almost inadvertently from *E.coli* and never again from an environmental source, and numerous genetic tools have been derived from this unique virus (Hendrix and Casjens, 2006). Transduction, described by Lederberg in the 1950's, was amongst the first techniques exploiting bacteriophage abilities to recombine with host DNA (Zinder and Lederberg, 1952). Since then, bacteriophage recombinases have been extensively studied leading to the classification of these enzymes into two families: the lambda integrase group (with a conserved tyrosine residue) and the serine recombinase group (with a conserved serine residue) which includes the phiC31 integrase (Smith et al., 2010). In both groups, some of these enzymes were further used to develop tools to manipulate genomes not only within their original host but also outside the bacterial world. For instance, recombination genes of Lambda led to the development of *in vitro* techniques to perform directed

gene replacement in fungi (Chaveroche, Ghigo, and d'Enfert, 2000). A recent development of the use of PhiC31 integrase in the long-term expression of transgenes in mammalian cells may become an important tool for human gene therapy (Chavez and Calos, 2011; Keravala et al., 2008). After the huge contribution of a very limited number of different bacteriophages in the unveiling of numerous molecular mechanisms conserved through cellular life, the current development of viral metagenomics is likely to increase the number of applications exploiting viral genes (Schoenfeld et al., 2010). In this article we review some of the most recent applications covering viral proteins, virion structures, full viruses and highlight some future directions.

2°) Exploiting the specificity

One of the main hallmarks of viruses is their specificity for their target. In many environments, viruses are exposed to a plethora of hosts and their capacity to infect only a limited number of them drives their population expansion and maintenance. This outstanding property has therefore been exploited in numerous applications among which bacteriophage typing was the first to be widely used. The susceptibility to different bacteriophages provides an easy way to classify closely related bacterial strains. Bacteriophage typing was introduced in the 1940's and despite the development of molecular techniques, it is still being used, for example, by the Health Protection Agency (UK) to type *Salmonella* and *Staphylococcus aureus* strains (http://www.hpa.org.uk/). This is in fact not so surprising since molecular techniques based on the presence / absence of DNA fragments do not take into consideration the expression of proteins coded by these fragments, as well as the activity of these proteins. In contrast, bacteriophages rely on the presence / absence of molecular motifs expressed at the surface of bacteria and remain useful tools to provide a rapid answer, generally within a day, which is valuable in an outbreak situation (Sechter, Mestre, and Hansen, 2000). However, it may not be long before the price of the latest sequencing technologies drops to the point where a full genome sequence will become available within a couple of days, thus rendering obsolete the bacteriophage typing activity.

It is clear that the rapid and accurate identification of bacterial pathogens is vital in order to prevent the spread of infections and limit their consequences by implementing appropriate control strategies. This is a particularly important concern for hospitals, care centres and in the food industry. Several bacteriophages have been exploited for technology development to enable the rapid detection of bacteria in complex environments, in

particular through the use of bacteriophage based probes (Hagens and Loessner, 2007; Rees and Dodd, 2006; Ripp, 2010; Singh et al., 2012; Smartt et al., 2012). However, very few diagnostic kits for the detection of pathogens in human samples such as the FASTPlaqueTB assay and the MRSA/MSSA Blood Culture Test have yet reached the market (Stanley et al., 2007), (http://www.microphage.com/technology/). The current applications of bacteriophages in pathogen detection, can be divided into two groups: those based on inert bacteriophage particles or isolated bacteriophage proteins and those that require an on-going infectious cycle. Both systems have been developed targeting *E.coli* 0157:H7, a major food pathogen. One of them, based on the specificity of a bacteriophage tail protein, is already available on the market (VIDAS UP E. coli O157, Biomérieux). Briefly, in this assay, a bacteriophage specific for *E. coli* 0157:H7 was isolated, its tail fiber protein was identified and produced as a recombinant peptide, labelled with a fluorescent dye and included into the VIDAS sandwich assay technology. Another ingenious device using infective particles this time was also designed. Combining horseradish peroxidase-labelled bacteriophage with a serogroup-specific immunomagnetic separation system and enrichment broth, it allows pre-amplification of the pathogen before its detection by a luminometer (Willford et al., 2011). Among the viral proteins exploited for the detection of hosts, we can distinguish between tail fiber proteins as mentioned above, required for the early step of the infectious cycle, and endolysins used for bacteriophage release, the last step of the cycle. The use of tail fiber proteins relies on the specificity of a bacteriophage protein towards its bacterial receptor. Other examples of such proteins in pathogen detection include the receptor binding protein (RBP) of a *Campylobacter* bacteriophage and the P22 tailspike protein conjugated to nanoaggregate embedded beads (Tay et al., 2012).

Endolysins, participate in the lysis of the host cell by degrading specific bonds within the bacterial peptidoglycan in order for the bacteriophage progeny to be released at the end of the lytic cycle. Endolysins reported to date are either globular or modular. Modular endolysins display at least one N-terminal catalytic domain and a C-terminal cell wall binding domain (Fischetti, 2008; Loessner, 2005). The cell wall binding domain (CBD) of a specific endolysin shows high specificity and affinity for its ligand on the cell wall of its host, readily accessible in Gram positive bacteria. This specificity has been exploited in various techniques, in particular for detection. One example is an assay in which CBDs of *Listeria monocytogenes*, *Bacillus cereus* or

Clostridium perfringens were coated onto paramagnetic beads, allowing a better separation and recovery of bacterial cells than with standard techniques (Kretzer et al., 2007).

Applications requiring an infectious particle may use labelled particles as described above but may also be based on genetically modified bacteriophages leading to the expression of a reporter gene that is highly expressed upon bacteriophage infection. Examples of these reporter bacteriophages include those carrying luminescence genes such as, the Listeria bacteriophage A511 in which *Vibrio harveyi luxAB* genes were inserted immediately downstream of the gene encoding the major capsid protein, or bacteriophages for *Yersinia pestis* and *Bacillus anthracis* in which a similar approach proved efficient in detecting these two pathogens, whose rapid diagnosis is crucial in the fight against bioterrorism (Loessner et al., 1996; Schofield, Molineux, and Westwater, 2011). Other types of reporter genes can also be inserted in bacteriophage genomes, such as a hyperthermophilic β-glycosidase gene used for the detection of Listeria cells (Hagens et al., 2011). The main advantage of the latter is that the overtime signal amplification goes on as long as the substrate is provided, long after the host has been lysed.

Finally, one may simply use the lytic nature of the bacteriophage as the "sensor" to detect of the presence of the host. This is the principle of the *FASTPlaque*TB assay for the detection of *Mycobacterium tuberculosis* in sputum in which bacteriophages infecting the slow-growing tubercle bacillus are subsequently detected when forming plaques on a fast-growing strain (Rees and Loessner, 2005). This technique can also be coupled to a molecular tool for higher specificity as in the phage-IS900 technique used to detect viable *Mycobacterium avium paratuberculosis* in dairy products, a pathogen thought to have a role in Crohn's disease pathogenesis (Botsaris et al., 2010).

By definition, these detection systems are designed to be disposable and single use kits, therefore the nature of the key components (sometimes genetically modified) does present safety issues, and their usage will have to be constrained within an appropriate waste management protocol. In addition, these systems often developed for one particular host can be adapted for many others upon identification of the corresponding genes or proteins in bacteriophage genomes, which nevertheless, can be sometimes difficult when working with a new bacteriophage (typically a new bacteriophage possesses over 70% of ORFs that do not match any ORFS present in genomic databank)

Apart from pathogen detection applications, the viral specificity may also be used for targeted delivery such as gene delivery to a target cell for therapeutic purposes. Genetically engineered bacteriophages used as vectors for nucleic acids may target bacterial or eukaryotic cells. For example, non-lytic filamentous bacteriophages can be used to deliver genes encoding proteins that are toxic to their bacterial host, such as the delivery of "addiction toxins" triggering programmed cell death via M13 phagemid system (Hagens and Blasi, 2003; Westwater et al., 2003). The use of a non-lytic bacteriophage presents the advantage of killing the bacterial cell while leaving it intact, thereby limiting the release of bacterial endotoxins and decreasing the resulting inflammation (Hagens et al., 2004).

Among virus-derived applications of gene delivery to eukaryotic cells, one can cite adeno-associated virus AAV works towards therapy for lung cancer via the delivery of the *CD40L* gene, or studies using Herpes-simplex virus for the transfer of the C3 transferase gene to indirectly promote axonal regeneration (Wu et al., 2007; Zhou et al., 2012b). Bacteriophages also have a potential in gene therapy via transfection of genes toxic for cancer cells. This could be a promising future strategy for treating advanced lung and colon cancer, combining gene transfer with chemotherapeutic agents (Rama et al., 2011).

3°) Exploiting bacteriophages for biocontrol purposes

In the environment, viruses play a major role as biocontrol agents for their hosts and do so through either one of the two life cycle strategies they use. A bacteriophage will either infect its host to immediately produce its progeny leading to the death of the host (virulent cycle), or it may delay the progeny production by staying silent within its host genome for a number of generations (temperate cycle). For obvious reasons, bacteriophages displaying a temperate nature are not suitable for use in the development of biocontrol strategies. Strictly lytic viruses, however, are very much appropriate for biocontrol downstream applications. The process of infecting and efficiently killing their bacterial hosts has been refined over billions of years of evolution and shows a great versatility in overcoming possible resistance mechanisms that a bacterium could evolve (Labrie, Samson, and Moineau, 2010). This type of bacteriophage has indeed been broadly exploited for the biocontrol of bacteria for human, industrial or ecological interests.

The alarming problem of the increasing occurrence of antibiotic resistant bacteria has recently revived the interest in bacteriophage therapy. Despite the fact that human bacteriophage treatment has been carried out over the last 80 years in Eastern Europe, the evolution of safety and regulation standards in Western countries during the last 30 years is currently preventing the development of large scale clinical trials due to the lack of recent data on both efficacy and safety (Maura and Debarbieux, 2011). Nevertheless, within the last decade, an increasing number of proof of concept studies were achieved using animal models. As for human testing, one phase II clinical trial using the Biophage-PA bacteriophage preparation towards antibiotic-resistant *Pseudomonas aeruginosa* in 24 patients with chronic otitis was published, concluding to its safety and efficacy (Wright et al., 2009). Another trial, supported by the Nestlé Nutrition Corporate and involving the oral administration of T4 bacteriophages in young children with diarrhea due to ETEC/EPEC infections, is currently being carried out and is due to completion in 2014 (Clinical Trial Identifier NCT00937274).

Biocontrol of pathogens in food products may represent an economically viable field for bacteriophage-based biocontrol. This topic was extensively reviewed (Garcia et al., 2008; Goodridge and Bisha, 2011; Hagens and Loessner, 2010; Mahony et al., 2011). Many studies reported success in decreasing the bacterial load (for *Listeria*, *Salmonella* and *E. coli* species among others) on food surfaces (cheese and meat), hard surfaces and also cattle hide (Anany et al., 2011; Coffey et al., 2011; Guenther et al., 2012; Viazis et al., 2011; Viscardi et al., 2008). Several systems have already been approved by authorities for use on food products such as ListShield™ (Intralytix) or LISTEX™ (Micreos) for the control of *L. monocytogenes* (both FDA- and USDA-approved), EcoShield™ (FDA-cleared) targeting *E. coli* O157:H7 and SALMONELEX™ against *Salmonella* which also beneficiated from a Temporary Use Exemption by the Dutch Medicine Evaluation Board for the currently ongoing field trials. Bacteriophages were also found to be efficient for the decontamination of livestock raised for the food industry, therefore limiting the risk of pathogens reaching the food chain, or to detect the presence of pathogens in animal-derived alimentary products. Bacteriophages have shown success in control of plant pathogens too with recent examples including the development of a bacteriophage-based biocontrol technique for *Dickeya solani*, a bacterium which causes potato plants to waste, and the effective prevention of wilting using a cocktail of three lytic bacteriophages for the bacterium *Ralstonia solanacearum* (Adriaenssens et al., 2012; Fujiwara et al., 2011). The company Omnilytics developed the bacteriophage

product “Agriphage” for the control of bacterial spot caused by *Xanthomonas campestris* or bacterial speck caused by *Pseudomonas syringae* (www.omnilytics.com). The effectiveness of this product in protecting the crops against these pathogens is reflected by the report of a marked increase in yield. However, early suggestions that bacteriophage interaction with plants might actually decrease plant growth or not show improvements compared to regular treatments are also reported, highlighting the need for further evaluation of possible biocontrol strategies before implementation is considered (Gill and Abedon, 2003; Jones et al., 2007; Kocharunchitt, Ross, and McNeil, 2009; Ye et al., 2009).

Finally bacteriophages are currently being investigated for their potential as natural biocontrol agents for environmental issues. Their natural presence in the environment would suggest a lower risk of natural ecology disturbance, making them an attractive alternative to chemicals as antibacterial agents. In this field, works have been carried out in the context of wastewater treatment and sludge processing. Only a few studies have been conducted so far but encouraging results in foam, sludge volume or biomass bulking reduction were reported (Choi, Kotay, and Goel, 2011; Kotay et al., 2011; Petrovski, Tillett, and Seviour, 2012).

Besides the use of entire bacteriophage particles, the use of isolated proteins has also raised interest lately. The use of single gene products by-passes the difficulties of using infective particles and tends to reassure the community by eliminating the risk of genetic recombination and general instability linked to the idea of using viruses while keeping the benefit of the main hallmark of the bacteriophage, that is to say its specificity.

As mentioned earlier, endolysins cleave peptidoglycan bonds enabling them to lyse Gram positive bacteria when applied externally. The potential use of these proteins as antimicrobial agents has been extensively reviewed (Coffey et al., 2010; Fenton, 2010; Fischetti, 2008; Fischetti, 2010; O'Mahony et al., 2011). More recently, the production of recombinant lysins (engineered or chimeric lysins) has also been achieved in a number of studies (Pastagia et al., 2011; Resch, Moreillon, and Fischetti, 2011; Schmelcher et al., 2012). Their antimicrobial potential against *S. aureus*, *B. anthracis*, *Streptococcus pneumoniae*, *Enterococci*, *L. monocytogenes* or *Clostridium difficile* to cite only a few, is well documented (Kikkawa et al., 2008; Mayer, Narbad, and Gasson, 2008; Rashel et al., 2007; Rodriguez-Cerrato et al., 2007; Yoong et al., 2004; Zhang et al., 2012a). Most recent advances in the field of chimeric lysins led to the design of a hybrid protein targeting Gram negative pathogens, whose structure is templated on a bacterial toxin called pesticin. It is composed of T4

lysozyme fused to the domain of pesticin targeting FyuA, an outer membrane transporter but also a major virulence factor for some *Yersinia* and pathogenic *E. coli* strains. This hybrid lysin has therefore the potential to cross the outer membrane of pathogens to gain access to the peptidoglycan, and was shown to be able to kill specific *Yersinia* and pathogenic *E. coli* strains (Lukacik et al., 2012). Following infection, the bacteriophage quickly hijacks the host cellular machinery to its own profit in order to redirect it to the production of the proteins that will constitute its progeny. To do so more efficiently, bacteriophages often encode early proteins inhibiting the processing of the host proteins. Several of these early proteins have been identified by different groups and their implication in antibacterial compounds investigation has been reviewed by Sau et al, 2008 (Sau et al., 2008). Examples of such proteins include peptides inhibiting bacterial replication by interacting with the host DNA replication proteins, peptides inhibiting the cell wall synthesis such as the Kil proteins in coliphages Mu or Lambda and peptides interfering with bacterial transcription by inhibiting the synthesis of the host proteins however preserving the synthesis of their own (Nechaev and Severinov, 2008).

When the bacteriophage protein does not have a bactericidal action in itself, it can also be fused to a bactericidal agent in order to direct the latter to its target. This approach was used successfully by Scholl et al., who fused the O-antigen-specific bacteriophage tail spike gene identified in the genome of the O104:H4 *E. coli* strain, to a *Pseudomonas* pyocin to obtain an engineered bacteriocin displaying a specific activity against the O104:H4 serotype (Scholl et al., 2012).

4°) Exploitation of bacteriophage structural properties

Due to their high level of organisation, viruses represent elaborate nanomachines. In particular, filamentous bacteriophages, that can be easily genetically manipulated, have been the focus of many studies and allowed the development of bacteriophage display techniques (Figure 2). An increasing number of applications in the field of nanotechnology is driving many researchers to exploit bacteriophage properties among which we can distinguish their use as vectors or as matrices for new nanotechnological devices with a wide array of applications in nanomedicine (Fan, Chen, and Xie, 2012; Hemminga et al., 2010; Hyman, 2012; Rakonjac et al., 2011).

A. Viral/Phage display.

Phage display, the principles of which are detailed in several reviews involves the engineering of the bacteriophage in order for the virus to carry foreign molecules on its surface through translational fusion with a surface protein, or through conjugation techniques (Pande, Szewczyk, and Grover, 2010; Sidhu et al., 2000).

The use of a bacteriophage to display foreign peptides was first reported in 1985 by Smith and since then, the relevance of this now famous system has found numerous applications in different domains including epitope identification, antigen delivery, drug discovery, vaccine design, targeting of eukaryotic cells, bioimaging and biosensing, enzyme display or nanomaterials design (Adda et al., 2002; Ahmadvand, Rahbarizadeh, and Moghimi, 2011; Collins et al., 2006; Gao et al., 2010; Li et al., 2010; Molek, Strukelj, and Bratkovic, 2011; Prisco and De Berardinis, 2012; Seker and Demir, 2011; Smith, 1985; Szardenings, 2003).

Numerous viral platforms are used for displaying molecules at the surface of a virus particle, either through molecular manipulation or through chemical modification. These include eukaryotic viruses systems such as the cowpea mosaic and potato X viruses, TMV, turnip yellow mosaic virus or bacteriophage based systems with M13, Qβ, Lambda, T7 or T4 (Banerjee et al., 2010; Chatterji et al., 2002; Dunn, 1995; Pande, Szewczyk, and Grover, 2010; Ren et al., 1996; Smith et al., 2009; Sokoloff et al., 2000; Steinmetz et al., 2010; Sternberg and Hoess, 1995; Zan et al., 2012).

1. Principles: display through molecular manipulation or chemical modification

Molecular engineering of the bacteriophage genome is widespread and has been implemented for display in four main bacteriophage systems: T4, T7, Lambda, and filamentous bacteriophages (Beghetto and Gargano, 2011; Gupta et al., 2003; Hemminga et al., 2010; Kehoe and Kay, 2005; Kurzepa et al., 2009; Meng, Zhang, and Ai, 2011; Rakonjac et al., 2011; Teesalu, Sugahara, and Ruoslahti, 2012). In this approach, the gene introduced into the bacteriophage genome codes for a peptide that is expressed as a protein fusion on the bacteriophage surface. This technique is widely used in numerous fields of science for its potential of large scale screening as the inserted genetic information can be randomly generated to produce a library, achieve targeted delivery to specific locations or study molecule interactions. This display approach has been extensively applied to the M13 filamentous bacteriophage. M13 is a 880nm long rod shaped, single-stranded

DNA virus. Its viral capsid is composed of 2700 copies of helically arranged major coat protein (pVIII) and 5-7 copies of pIII, pVI, pIX and pVII, located at either of its ends (Petrenko et al., 1996; Smith and Petrenko, 1997).

Since its beginnings, the phage display technique and its application within the framework of libraries allowed the screening of a wide array of molecules based on their specificity or binding affinity to various compounds and the selection of ligands to virtually any target. Briefly, the antigen of interest is immobilized on a surface and exposed to the engineered bacteriophage library. The bacteriophage particles displaying on their surface a molecule with affinity for the immobilized antigen will therefore bind and the rest of the library will be washed away. The bound fraction can be eluted and amplified resulting in a mixture of bacteriophage particles displaying the relevant molecule. This procedure is repeated in a process called 'biopanning' and allows the identification of molecules with high binding affinity to the antigen of interest. This technique is widely applied and resulted in the identification of numerous molecules of interest with affinity to other peptides, nucleic acid fragments, carbohydrates but also plastic and polystyrene, or inorganic substances with interesting physicochemical properties (Adey et al., 1995; Vodnik, Strukelj, and Lunder, 2012)

However, the bacteriophage library concept also presents limitations. For example, the amplification of the libraries during which bacteriophage candidates are amplified in the bacteria, leads to a certain level of competition between numerous bacteriophage clones thus resulting in the loss of library diversity. This therefore restricts the number of ligands that can be identified in one screen and may represent a severe constraint for targets with multiple binding sites (Derda et al., 2011).

One should also bear in mind that the displayed peptide size and charge should be taken into account as they influence the number of peptides that can be displayed on the surface of a virion. In order to retain a good bacteriophage production during amplification, the normal processing of the bacteriophage coat protein should indeed not be hindered by the fused peptide in order to allow its correct expression and to let the protein fulfil it biological role in the bacteriophage replication process (Imai et al., 2008). Following these observations, Merzlyak and Lee published a method using degenerate flanking residues, to express functional but unfavorable peptide motifs on major coat protein pVIII of bacteriophage M13 while limiting the bacterial biological

censorship involved in the natural pVIII protein processing during the bacteriophage replication (Merzlyak and Lee, 2009).

Alternatively, viruses can also be "decorated" with molecules by means of chemical modification in a process called bioconjugation (Gillitzer et al., 2002). This technique has been utilized for the binding of peptides (CPMV), polymers (adenoviruses), carbohydrates (CPMV) but also proteins and nucleic acids via surface functionalization as for MS2 and Qβ bacteriophages capsids (Chatterji et al., 2004; O'Riordan et al., 1999; Patel and Swartz, 2011; Raja, Wang, and Finn, 2003; Sen Gupta et al., 2005). Binding of inorganic molecules such as gadolinium chelate groups (MS2 bacteriophage capsid) was also achieved (Anderson et al., 2006). This approach allows the attachment of molecules on the outside but also the decoration of the inside of the protein shell, for example for the use of viral particles for cargo transport (Kovacs et al., 2007). The use of archaeal viruses, such as SIRV2 whose surface can also be functionalized, represents an interesting option due to the extremophile nature of the hosts conferring these viruses with an outstanding stability in harsh physical conditions (temperature, pH…), making them attractive for applications in nanotechnologies (Steinmetz et al., 2008).

2. Main Applications

Since the display technique was discovered, a tremendous amount of studies have been carried out (Figure 2). One can observe a marked increase in the published literature for twenty years and then, the plateau could suggest that we may have reached a limit in the number of new applications derived from this technique. This approach is now used in various domains of science such as in antiviral research, novel therapeutics and vaccine design, cancer research, targeted delivery, imaging or nanotechnologies to cite only a few ranging from biology to nanotechnologies (Castel et al., 2011; Gao et al., 2010; Huang, Bishop-Hurley, and Cooper, 2012; Hyman, 2012; Li et al., 2010; Ronca et al., 2012; Staquicini et al., 2010; Yamashita et al., 2010). Exhaustive information can be found in numerous articles, book chapters or scientific reviews on the technique itself (Bratkovic, 2010; Miller, Vandome, and McBrewster, 2010; Rentero and Heinis, 2011; Smith and Petrenko, 1997; Vodnik et al., 2011). A selection of applications from different domains is listed in Table 1.

The display technique involving chemical conjugation is widely used for detection purposes through the binding of contrast agents, of biotin-streptavidin systems for conjugation of peptide-displaying bacteriophages to quantum dots or of fluorescent dyes for bioimaging or biosensing purposes (Anderson et al., 2006; Edgar et al., 2006; Li et al., 2010). To cite one example, T4 heads were used as scaffolds for the conjugation of Cy3 and Alexa Fluor 546 fluorescent dyes in order to design virus based nanoparticles suitable for cell imaging (Robertson et al., 2011). This conjugation technique can also be applied to proteins after surface functionalization as demonstrated for MS2 and Qβ VLPs or to DNA as shown for M13 bacteriophage (Lee, Domaille, and Cha, 2012; Patel and Swartz, 2011). In some cases, molecular engineering of the bacteriophage genome and chemical modification of its surface can be coupled, as previously demonstrated for EGF-displaying Qβ particles subsequently conjugated with a fluorescent dye for visualization of cellular attachment and uptake monitoring (Pokorski, Hovlid, and Finn, 2011).

3. Bacteriophages and nanotechnologies

Peptide display by filamentous bacteriophage allowed the identification of molecules able to bind to inorganic materials and eventually, peptides with selectivity for binding to metal surfaces (Brown, 1997). Works by the team of A. M. Belcher show how phage-display libraries can be used to obtain peptides that bind to a range of semiconductor surfaces with high specificity, depending on the crystallographic orientation and composition of the material, in the view of using this principle with materials important in electronics or optics (Whaley et al., 2000). Molecules with such affinities as well as the highly organised structure of the bacteriophage particle are elements extensively exploited in the construction of periodically ordered nanomaterials such as nanometre-scale electronic and medical materials. This resulted in the design of a model for the fabrication of a highly ordered composite material from genetically engineered M13 bacteriophage and zinc sulfide nanocrystals (Lee et al., 2002). This led to biotechnological advances as varied as the “creation” of films, fibers, or nanowires with applications in the assembly of materials for lithium ion battery electrodes, photochemical devices and even improved catalysts through the templating of nickel, rhodium, and ceria onto the surface of the M13 bacteriophage (Huang et al., 2005; Lee et al., 2009; Mao et al., 2003; Mao et al., 2004; Merzlyak, Indrakanti, and Lee, 2009; Nam et al., 2006; Nam et al., 2008; Nam et al., 2010a; Nam et al., 2010b; Neltner et al., 2010).

Most recently published works on genetically engineered M13 describe the possibility of generating piezoelectric energy as well as nanorods and nanowires ranging from 300 nm to micron sizes which may find uses in nanoelectronics, sensing, and cancer therapy (Lee et al., 2012; Zhou et al., 2012a).

In addition, inorganic molecules assembled into electronic and magnetic particles led to the construction of biomedically relevant materials such as artificial bone substance created through the mineralization of a nanofiber constituted bundle of M13 bacteriophage or liquid-crystalline film matrices able to control and guide cell behaviour for tissue-regenerating applications (Chung et al., 2010; He et al., 2010; Merzlyak, Indrakanti, and Lee, 2009; Xu et al., 2011).

B. Bacteriophage proteins/scaffold

1. Cargo

The bacteriophage capsid is built from a number of protein subunits assembled together. Already extensively used for the display of molecules or material assembly, this protein scaffold can also be used for its structure i.e. as a cage to entrap biomolecules of interest. An ideal construct needs to deliver relevant biomolecules on command to a specific target while avoiding the neutralization by the immune system. Several of these steps have already been achieved by different groups. For example, the modification of the internal and external surfaces of the bacteriophage MS2 capsid to turn it into an efficient drug delivery system was described (Kovacs et al., 2007). In this particular study, first, extensive PEGylation of the capsids effectively reduced the neutralization by antibodies. Second, the binding of a ligand (biotin for example) to the distal ends of the polymer chains (PEG) indicated the possibility to attach targeting molecules to direct the cargo to a specific target. This approach could be applied to various ligands with specific tissue targeting properties (Weissleder et al., 2005). Third, the encapsidation of large “druglike” molecules was achieved. The synthesis of such modified particles demonstrates the potential of this system for efficient drug delivery. More recently, another study reported the synthesis of MS2 particles that could specifically deliver a number of molecules such as nanoparticles, drugs, siRNAs or toxins to hepatocellular carcinoma cells via the co-display of a targeting peptide and a fusogenic peptide promoting endosomal escape (Ashley et al., 2011). Finally, controlled release

of a high-copy cargo from engineered P22 capsids through morphological changes induced by a change in temperature was described (O'Neil et al., 2011).

2. Phi29 motor

Phi29 is another example of a virus which has been extensively used in the field of nanosciences (Zhang et al., 2012b). More precisely, the DNA packaging motor of Phi29, required to package double stranded DNA into the procapsid, has been studied in depth at the molecular level. This motor that uses ATP to push DNA into the procapsid requires only three components, the gp10 (connector) and gp16 proteins and a small packaging RNA (pRNA). *In vitro* association of purified recombinant proteins and artificially synthesized RNA was sufficient to reconstitute an active motor. This system led to the development of a wide variety of applications as the bionanomotor can be turned on and off on demand (absence/presence of ATP). In addition, not only the motor itself but its individual components such as the connector and the pRNA present molecular characteristics (oligomerization) for which several applications in nanotechnologies have been proposed (Ye et al., 2012).

5°) Perspectives

In half a century of research on viral-derived applications, many molecular tools were developed that helped decipher fundamental biological processes. Following this "golden age" (Figure 1), the scientific community's interest shifted towards "more complex" systems. However, in the past decade, two main fields have revitalized the interest for viruses-derived approaches. On one hand, the advent of nanotechnology that showed many promises based on purely chemical processes is now diversifying towards bionanotechnology for which viruses have been the first biological entities to be used. On the other hand, the impressive reduction in the cost of DNA sequencing now allows the production of a massive amount of data from the analysis of environmental samples. The main hurdle to overcome for the identification of viruses from genomic data is the presence of many unknown sequences within new viral genomes, currently preventing an accurate assembly of short reads. These data are only starting to be exploited for the deciphering of viral variety and progressively uncovering the extent of its diversity. Analysis of these metagenomic studies vastly broadens our current knowledge on viral specimens and lifestyle (Krupovic and Forterre, 2011). Sequence assembly issues resulting from short reads

should shortly be solved by the next generations of sequencing technologies. Meanwhile, much can be learned from proteins identified with improved techniques and through the presence of conserved domains such as in DNA polymerases or endolysins (Schoenfeld et al., 2010). However if a virus has evolved with a different mechanism from those already known, there is a risk that it will be missed by this approach. While these two domains are still being developed, they carry the potential to increase the number of potential virus-derived applications. For example, DNA polymerases with new combination of properties (proofreading, thermostability, helicase…) could be identified. It could also be anticipated that amongst new viruses identified by sequencing, some display enough homology between regions comprising a number of unknown genes, to be grouped. Looking deeper within these conserved regions might suggest ORFs to be studied in priority for a given function, the easiest ones being those related to the hijacking of the host machinery as these proteins are not often specific to one particular host. As well as contributing to reduce the proportion of unknown genes within bacteriophage genomes, cloning these ORFs in model organisms such as *E. coli* or *S. aureus* could reveal new antibacterial activities whose mechanisms would inspire the elaboration of new drugs.

The recent discovery of giant viruses has revolutionized the vision of low complexity associated with viruses (Koonin and Yutin, 2010; Raoult et al., 2004). Carrying in their genome a large amount of genetic information coding for elements usually hijacked from the host, these viruses could become platforms to further develop new tools able to support a higher degree of complexity than bacteriophages, such as host-independent processes.

The rate of identification of previously unreported organisms increased markedly with the development of metagenomics, suggesting that numerous hosts and their viruses present in the environment are yet to be discovered. To identify them, the use of microfluidic PCR to link viruses to their hosts in environmental samples without cultivating them has been recently developed (Tadmor et al., 2011). In particular, this approach applied to the human gut could reveal the degree to which viruses and their hosts interplay in this complex polymicrobial environment. Sequencing the viriome of the human gut has already revealed the prominent contribution of prophages to genetic exchanges (Reyes et al., 2010; Reyes et al., 2012). In contrast, virulent bacteriophages appear underrepresented suggesting that the gut environment may be unfavourable to them. On the other hand, data obtained in a mouse model showed continuous replication of virulent

bacteriophages over several weeks suggesting a high level of complexity in the relationship between bacteriophage and host populations in the gut environment (Maura et al., 2011). Several studies have also shown that *in vitro* polymicrobial biofilms can be reduced by bacteriophages (Brussow, 2012; Donlan, 2009; Siringan et al., 2011). However these systems are not yet similar enough to real situations, such as in medical and environmental settings, to claim that bacteriophages can efficiently reduce biofilms on their own. One solution may come from a combinatorial approach where a bacteriophage infecting one species may also code for hydrolytic enzymes targeting the extracellular matrix produced by this species or others (Lu and Collins, 2007). Such complex environments including various cellular types represent a challenge for viruses and one may ask whether, to some extent, this lifestyle developed, as a form of resistance to viral attack.

Combination of activities might be a future direction in which viruses-derived tools versatility could play an important role. In particular, one can imagine designing a virus targeting for example a gram negative host and carrying genes that are toxic for gram positive species (such as endolysins) or fungi. Coupling these different properties may offer the possibility to come up with new highly efficient broad antimicrobial strategies. With the advent of *in vitro* synthesis of small genomes such approaches are theoretically feasible and, according to progress in synthetic biology, could have a broader impact than currently appraised on the development of future applications (Wimmer et al., 2009). However, there are two main limitations to such a concept: 1) an extensive knowledge on viruses intended to be used as platforms should be achieved; 2) specific guidelines should be defined to regulate the use of these viral recombinants. Nevertheless, it might be possible that such chimeric viruses exist or have existed in nature at some point but have been lost due to evolutionary pressure. The dynamics of the viral world are indeed so high that, to date not a single gene has been identified that is conserved amongst all bacteriophages sequenced (Liu, Glazko, and Mushegian, 2006). Their genome plasticity, one of the trademarks of viruses, is such that they are continuously creating new answers to the recurring problem of the necessity of infecting their host, thus representing a source of pressure for biological innovations that humans are trying to exploit for their own interest.

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Table 1: Main applications of Phage display

	Principle	**Putative application***	**Reference**	**Bacteriohage**
Peptide identification	Identification of tumour homing peptides Eg RGD-4C Peptide (cancer cell targeting)	Tumour cell targeting,	(Pasqualini and Ruoslahti, 1996; Rivinoja and Laakkonen, 2011) (Arap, Pasqualini, and Ruoslahti, 1998)	M13 / T7
	Identification of organ-homing peptides Eg PSMA (prostate-specific membrane antigen) or TGN (peptide targeting the drug delivery system to the brain)	Organ targeting Antigen detection Targeted drug delivery across the blood brain barrier when bound to nanoparticles	(Pasqualini, 1999) (Arap et al., 2002) (Arter et al., 2012) (Li et al., 2011)	M13
	Identification of white fat vasculature homing peptide	Control of obesity	(Kolonin et al., 2004)	M13
	Identification skin/membrane-permeable peptide	Alzheimer's therapy	(Zhang et al., 2010)	
	Identification of skin penetrating and cell entering peptide	Treatment of various skin diseases	(Hsu and Mitragotri, 2011)	M13
	Identification of peptides binding to organic crystals	Modification of surface chemistry of the crystals – application in agriculture	(Cho, Fowler, and Furst, 2012)	M13
	Identification of short peptides mimicking carbohydrates conformation, lectin mimics	Glycobiology – Anti-carbohydrate antibodies production – discovery of biomimetic peptides against carbohydrate and lectin targets	(Fukuda, 2012) (Gerlach et al., 2010) (Yu et al., 2009)	M13

	Identification of peptides able to bind to inorganic materials	Applications in electronics and opticals – bacteriohage-based electrodes and batteries -nanotechnologies	(Whaley et al., 2000) (Nam et al., 2006; Nam et al., 2008)	M13
Peptide display	Display of antigenic peptides Eg the EFRH epitope Integrin-binding peptides Display of specific antigen binding peptides (PSMA-specific peptide)	Peptide Vaccines design Elicits humoral response in Alzheimer's patients Tissue-regeneration matrices Biodetection by bacteriophage-based electrodes	(Gao et al., 2010) (Solomon, 2008) (Chung et al., 2010) (Yang et al., 2006)	T4, T7, Lambda, filamentous bacteriophages
	Bacteriohage probes	Detection of viral and bacterial pathogens	(Goldman et al., 2000; Petrenko and Sorokulova, 2004)	
	Dye-labelled peptide-displaying bacteriophages	Sensing of environmental pollutants	(Goldman et al., 2002)	M13
Peptide delivery	Display of a peptide inducing endocytosis and a peptide interfering with the pathogen	Potential therapeutic strategy to prevent or reduce sexually transmissible diseases	(Bhattarai et al., 2012)	M13
Display of antibody or antibody fragments	Peptide delivery coupled to display of an antibody / antibody fragment	Targeted antigen delivery system to professional APCs - application in new vaccines design	(Prisco and De Berardinis, 2012)	fd
	Display of cocaine-sequestering antibodies	Drug addiction control	(Dickerson, Kaufmann, and Janda, 2005)	Not specified, no full text
	Display of engineered	Alzheimer's disease therapy	(Solomon, 2008)	Filamentous

	monoclonal antibodies able to enter the CNS			bacteriophage
	Display of llama single-domain antibodies (sdAbs)	Drug transport across the BBB	(Muruganandam et al., 2002)	M13
	Display of antibody fragments binding cell surface receptors in a manner that leads to endocytosis	Entry into mammalian cells, targeted gene delivery	(Poul and Marks, 1999)	F5
	Display of antibody variable region	Production of recombinant anti-carbohydrate antibodies	(Fukuda, 2012)	M13
	Display of antibody fragments against viruses	Tools for diagnosis and serotyping of dengue	(Cabezas et al., 2009)	??
Carbohydrate display	Display of HIV-related glycans	HIV glycan-based vaccine design	(Astronomo et al., 2010)	Qβ
Enzyme display	Display of alpha-amylase and xylanase enzymes	De-inking of office waste	(Zachariou, Widdowson, and Straffon, 2006)	T7
	Display of biofilm-degrading enzymes	Biofilm degradation	(Collins et al., 2006)	Various

Figures legend:

Figure 1: Rise and fall of bacteriophage publications over a century (search in Pubmed performed in June 2012 and percentages calculated from the number of hits bearing the term “phage” over 10 year periods).

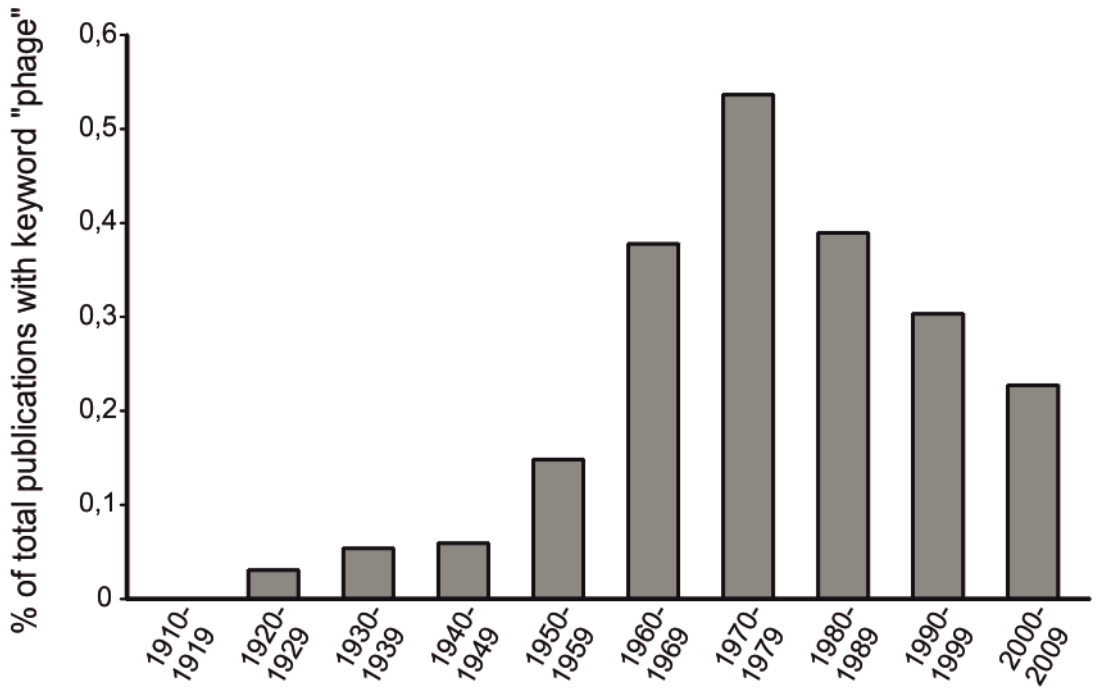

Figure 2: Rising of phage display and bacteriophage therapy related publications (percentages of the total “phage” publications dedicated to phage display and bacteriophage therapy related topics over the past two decades). (Search in Pubmed performed in September 2012 and percentages calculated from the number of hits bearing the terms “phage display”, or “phage” together with “therapy”).