Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Genetically modified bacteriophages in applied microbiology

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Keywords
bacteriophages, biopharmaceuticals, biotechnology, enzybiotics, genetically modified bacteriophages, pathogen detection, phage therapy.

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2016/0219: received 3 February 2016, revised 7 June 2016 and accepted 16 June 2016

doi:10.1111/jam.13207

Summary

Bacteriophages represent a simple viral model of basic research with many possibilities for practical application. Due to their ability to infect and kill bacteria, their potential in the treatment of bacterial infection has been examined since their discovery. With advances in molecular biology and gene engineering, the phage application spectrum has been expanded to various medical and biotechnological fields. The construction of bacteriophages with an extended host range or longer viability in the mammalian bloodstream enhances their potential as an alternative to conventional antibiotic treatment. Insertion of active depolymerase genes to their genomes can enforce the biofilm disposal. They can also be engineered to transfer various compounds to the eukaryotic organisms and the bacterial culture, applicable for the vaccine, drug or gene delivery. Phage recombinant lytic enzymes can be applied as enzybiotics in medicine as well as in biotechnology for pathogen detection or programmed cell death in bacterial expression strains. Besides, modified bacteriophages with high specificity can be applied as bioprobes in detection tools to estimate the presence of pathogens in food industry, or utilized in the control of food-borne pathogens as part of the constructed phage-based biosorbents.

Introduction

Bacteriophages (phages in short) are the most numerous biological entity on Earth with an estimated number of over 10^{30} phage particles (Brüssow and Hendrix 2002). They were independently discovered by Félix d'Hérelle and Frederick Twort in the early 20th century and shortly after they were harnessed in the treatment of bacterial infections (known by the term 'phage therapy'). Phage therapy back then struggled with mixed success, mainly due to a poor understanding of phage biology. The discovery of antibiotics led to the abandonment of phage therapy in the West, and thus, the development of phage-based products for medical purposes was restricted to few countries of the former Soviet Union and Eastern Bloc. Nowadays, when the problem of antibiotic resistance is becoming increasingly urgent, phages have been reconsidered as an alternative for the treatment of bacterial infections. The unique ability of phages to identify living bacterial hosts was not only utilized for medical purposes but soon found its way into bacterial diagnostics, where bacterial strains are differentiated on the basis of phage susceptibility. Phage typing has proven to be very sensitive; however, it could not be applied massively in the routine laboratory testing as it was labour intensive and time-consuming and as such has been replaced by molecular diagnostic methods.

With the advent of molecular biology and due to its simple lifestyle and structure of the genome, the phage has become a viral model of basic research (Keen 2015). The process of transduction where segments of bacterial DNA are transferred by the phage between the bacteria was utilized in genetic studies for constructing hybrid strains and subsequent gene mapping (Sanderson *et al.* 1972). The encounter of gene engineering with phages led to genetic modification of phage particles themselves. One of the most important techniques of phage modification is the phage display, originally designed by Smith (1985). The principle of this method is based on the fusion of phage coat protein genes with the genes encoding foreign peptides or protein fragments to create a large amount of phage variants displaying individual proteins on their surface. The phage displaying protein with the highest affinity to a target of interest is subsequently

identified by various selection steps. The yielded phage display library can vary, reaching over 10^{11} individual clones (Sblattero and Bradbury 2000).

The recombination of phage receptor-binding proteins, functionalizing of phages with active groups, and novel concepts of targeted delivery of genes by phages adds to the progress of phage engineering in the last years. The horizons of their practical application have been broadened from the original therapy through cancer diagnosis to the synthesis of nanomaterials.

In this review, we summarize current applications of genetically modified phages in applied microbiology. We focus on the treatment of bacterial infections and phage applications in biotechnology. Besides whole phage particles, attention is paid to the current protein engineering approaches to produce phage lytic enzymes for use in the treatment and diagnosis of pathogenic bacteria as well as in the control of biotechnological cultures.

Medical applications

Phage therapy

Today there are a number of phage-containing medical products available on the market. The Georgian companies Eliava Phages, Eliava Bio Preparations, and JSC Biochimpharm (Tbilisi, Georgia) and the Russian consortium Microgen (Moscow, Russian Federation) have developed several products for the treatment and prophylaxis of gastrointestinal infections, purulent infections or dysentery caused by all major staphylococcal, streptococcal and enterobacteriaceal pathogenic strains. However, these companies have only recently started to adhere to the international framework of Good Manufacturing Practices (GMP) in the fabrication of their products; therefore, none of these pharmaceuticals is currently available on the market in the Western world. An example of an already existing GMP product is Stafal®, a polyvalent phage-based drug manufactured by the company Bohemia Pharmaceuticals (Prague, Czech Republic), which is effective against methicillin-resistant *Staphylococcus aureus* (MRSA), available in Slovakia and under the specific treatment programme in the Czech Republic. The AmpliPhi Biosciences Corporation (San Diego, CA, USA) has two products ready to enter phase 1 and 2 clinical trials, which had been developed against infections caused by *Pseudomonas aeruginosa* and *Staph. aureus*. Besides the therapy products, development of a probiotic product containing phages against *Shigella* spp. has been announced by the company Intralytix, Inc. (Baltimore, MD, USA). In the last decade, the range of phages available for therapeutic purposes has been expanded with genetically modified variants.

Engineered phages with an extended host range

High specificity of host recognition is both advantage and disadvantage of phages in the mean of their medical application, as they avoid collateral damage to the normal human microflora, but require a precise diagnosis for the treatment to be successful. There are several lytic bacteriophages which have been well studied for clinical use against *Escherichia coli*, such as phage T4 or phage T2, both with a narrow host range. To construct a phage that would maintain a strong lytic ability and, at the same time, would infect an extended range of *E. coli* strains, homologous recombination between a long tail fibre gene region of phage T2 and the IP088 phage genes encoding tail fibres responsible for bacterial recognition was proposed, due to the wide infectious range of phage IP088 (Mahichi *et al.* 2009). The lysis assay confirmed the extended host spectrum of the recombinant phage without the loss of its lytic activity. In a similar study, long tail fibre proteins of phage T2 were replaced by the functional homologue of phage PP01, specific to pathogenic *E. coli* O157:H7 (Yoichi *et al.* 2005). The resulting construct had an identical host range to that of phage PP01; however, the obtained plaques were smaller than 0·5 mm in diameter, showing low efficacy of modified phage infection, with the emergence of resistant colonies.

The genes responsible for host recognition of phage T4 were also considered for the site-directed mutagenesis in the development of a virulent phage T4 bank active against a wide range of bacterial species, from *Yersinia ruckeri* to *Ps. aeruginosa* (Pouillot *et al.* 2010).

There are several other possibilities to extend the phage host range. It has been proven that genome rearrangements in gene encoding endolysin as well as tail structural genes can result in changes in the host range of polyvalent staphylococcal Twort-like phages (Kasparek *et al.* 2007). Strategy for engineering prophages to the lytic form for the species where no suitable lytic phage has yet been known was also investigated (Lynch *et al.* 2010).

Another option of extending phage host range is fusion of phage coat proteins with antibodies against a particular pathogen by phage display technology. Cao *et al.* (2000) fused single-chain variable fragments of a monoclonal antibody against *Helicobacter pylori* to the coat protein of phage M13. This system showed not only a high affinity towards *H. pylori* but also bactericidal activity *in vitro* and *in vivo* by a yet unknown mechanism. Phage display also represents a potential approach to combat infections caused by intracellular pathogens, as natural phages are not endocytosed by eukaryotic cells. Bhattarai *et al.* (2012) engineered phage M13 to display integrin-binding peptide on the major coat protein which

enables adhesion to eukaryotic cells and consequent integrin-mediated endocytosis. The minor coat protein of the phage was fused to specific peptide PmpD, a potential interfering agent, to control propagation of *Chlamydia trachomatis*, a widespread sexually transmitted intracellular pathogen. *In vitro* treatment of HeLa and primary endocervical cells with modified phage M13 led to a significant reduction in chlamydial infection. Such phage could be utilized in the fabrication of therapeutic materials. Already existing example is a polymeric film Phagobioderm® for treating wounds (Jikia *et al.* 2005).

Long-circulating phages

Merril *et al.* (1996) came up with a selection method for phages capable of circulating in the bloodstream of mammals for a longer period of time. They administered mice with phage λ and phage P22, sampled phages from the blood at 7 h postinfection, grew to high titres and re-administered the phages to another animal. After ten rounds of such selection, the phage concentration in the bloodstream at 7 h postinfection increased over 4 orders of magnitude for both phages. By genome sequencing, three new mutations were discovered in the selected phage λ compared to wild-type phage. A recombinant phage was subsequently prepared and the effect of a single substitution in a major coat protein on prolonged circulation was proven (Vitiello *et al.* 2005).

Circulation half-life of phages was considerably increased by chemical modification of their surface by polyethylene glycol (PEG). It was shown that PEGylated viruses reduce T-helper type 1 immune response, and phages are therefore more tolerated by mammals (Kim *et al.* 2008). For a successful treatment, achieving a high phage titre at the infection site is necessary. Furthermore, the distribution of a phage throughout the body and its persistence in the infected organs due to phage multiplication in pathogen represent a crucial issue for further enhancement of phage therapy (Górski *et al.* 2015). The idea of artificial phage displaying complement-antagonizing peptide, conferring higher phage resistance to the immune system, has also been proposed (Merril *et al.* 1997), but to our knowledge, no further research has been done in this area.

Biofilm disposal

Biofilm formation is a basic survival strategy of bacteria. A biofilm is a structure composed of a bacterial community producing a hydrated extracellular polymeric substances (EPS) matrix of a heterogeneous nature, consisting of polysaccharides, proteins and lipids. It protects bacteria against drugs, disinfectants, and various environmental influences by several mechanisms such as spatial protection and strong adhesion. Bacteriophages have been utilized against bacteria capable of biofilm production, responsible for serious complications in patients with cystic fibrosis or burns (Pires *et al.* 2011), and biofilms formed on medical implements in the hospitals from bacterial strains resistant to antibiotics (Donlan 2009). They pose a huge risk due to their high infectious potential, for example, during catheter insertion.

Lu and Collins (2007) came up with an idea based on engineered phages encoding hydrolases which are more active against EPS than naturally occurring phage enzymes (Fig. 1). They constructed a modified lytic phage encoding DspB, a glycoside hydrolase produced by *Actinobacillus actinomycetemcomitans*, responsible for biofilm disposal in its culture (Kaplan *et al.* 2003). The gene was placed under the control of the strong promoter T7φ10, ensuring its over-expression. *Escherichia coli* TG1, containing a couple of biofilm-promoting factors, was used

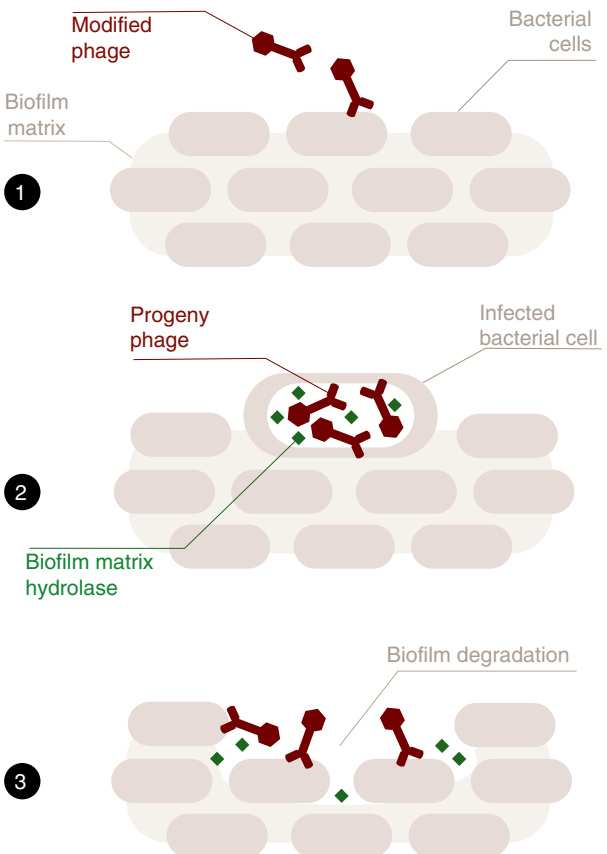

Figure 1 The principle of biofilm disposal using recombinant phages. After the infection (1), exponential multiplication of recombinant phages in the bacterial cells occurs together with over-expression of an active substance, encoded by the phages (2). The cells are lysed by phage progeny (capable of another infection) and the biofilm matrix is disposed by the hydrolase (3).

as a model organism. The yielded values clearly confirmed the efficacy of this system. Gladstone *et al.* (2012) suggested the possibility of a wild-type phage outgrowing the engineered variant in a mixed population, due to the benefit to the wild-type phage from a foreign gene product without the loss of energy necessary for its production. This was confirmed by evaluating the efficacy of phage T7DspB against long-term biofilms which was not statistically significant compared to that of the wild-type phage; nevertheless, the reduction of the biofilm biomass was still considerably high (Schmerer *et al.* 2014). Phage T7DspB thus proved to be an efficient tool for biofilm disposal and is already in development for commercial purposes by the company EnBiotix, Inc. (Cambridge, MA, USA).

Pei and Lamas-Samanamud (2014) constructed phage T7 encoding a lactonase enzyme that interferes with the quorum sensing mechanism of *Ps. aeruginosa* by degrading its signalling molecules, acyl homoserine lactones. Without the ability of communication, the bacterial population is not capable of forming biofilm. Their system was therefore predicted to be efficient in biofilm disposal, if this biofilm includes a natural host of phage T7 in which the phage could multiply. Besides being effective in biofilm reduction, this system also proved to be more efficient than the engineered *E. coli* culture, similarly over-expressing the lactonase, highlighting the effect of the phage itself in the disposal process.

Phages as delivery vehicles in medicine

The advent of the phage display technology enabled broadening the application range of the phage-based vectors in many medical fields. The lack of both toxicity and tropism towards eukaryotic cells combined with versatility, in which the phage particles can be modified, led to their prospect use in gene therapy, vaccine delivery and cancer *in vivo* imaging research (Hashemi *et al.* 2010; Robertson *et al.* 2011; Yata *et al.* 2014). Such range of application is possible as they can deliver both nucleic acids and proteins of interest either by replacing their genetic information with genes of interest (Westwater *et al.* 2003; Ou *et al.* 2013), fusing foreign proteins with their capsid protein (Tao *et al.* 2013; Liu *et al.* 2014), chemical functionalization of their coat (Robertson *et al.* 2011) or a combination of the above-mentioned strategies (Yacoby *et al.* 2007; Yata *et al.* 2014). By displaying endocytosis-mediating or cancer-specific peptides, tissue or cell-specific tropism of the phage vectors can be yielded (Yata *et al.* 2014). Efficacy of gene expression in eukaryotic cells can be further improved by fusing phage coat proteins with nuclear localization signals (Eguchi *et al.* 2005), re-circularization of the transferred DNA by Cre recombinase (Liu *et al.* 2014) or inserting the eukaryotic promoters to control the gene of interest (Clark and March 2004; Yata *et al.* 2014). Phage capsids alone along with packaging RNA of phage phi29 have also shown perspective as delivery vehicles (Robertson *et al.* 2011; Shu *et al.* 2013). In microbiology, phage-based vectors currently represent one of the fastest growing fields of applied bacteriophage research, with several spheres of utilization.

Delivery of lethal agents to pathogenic bacteria

After lysis of Gram-negative bacterial cells, a significant amount of lipopolysaccharide constituents of their outer membrane, that is, endotoxins, are released into the bloodstream of the infected host. The endotoxins are considered to be the most important bacterial factor in the pathogenesis of the Gram-negative septic syndrome, causing hemodynamic and metabolic derangements (Prins *et al.* 1994). Therefore, researchers aim at developing a treatment, which would kill bacteria without elevation of serum endotoxin level. It was demonstrated by Hagens and Bläsi (2003) that after administration of reporter phage, which does not trigger lysis of the cell, to a bacterial culture, only a negligible amount of endotoxins is released into the culture supernatant, comparing to a lytic phage. The killing mechanism of such reporter phage is based on the delivery of a gene encoding a bactericidal compound. By using phage T4LyD, unable to produce functional holin, Matsuda *et al.* (2005) have demonstrated *in vivo* clinical importance of this concept. The lysis-deficient killing phage was also designed against MRSA and achieved a higher survival rate in infected mice compared to antibiotics (Paul *et al.* 2011). A different approach of Embleton *et al.* (2005) consisted of phage-mediated transfer of light-activated antimicrobial agents—photosensitizers, generating cytotoxic singlet oxygen after exposure to light of a suitable wavelength. Although not genetically but chemically modified phages were used in this study, it proves the efficacy of the phage as a delivery vehicle in killing antibiotic-resistant bacteria.

Westwater *et al.* (2003) have come up with an idea of using a phage M13-derived vector system (phagemid) transferring bactericidal toxin to treat bacterial infection without causing pathogenic cell lysis. Phagemids contain both the phage and the plasmid origin of replication, with a foreign gene under the control of a strong promoter. To reproduce phagemids, co-infection with a helper phage encoding structural proteins is necessary, providing an advantage of reproduction control. Moradpour *et al.* (2009) have designed a phagemid intermediating a metabolism collapse of pathogenic *E. coli* O157:H7,

by delivery of the modified transcriptional regulator CAP, normally responsible for the activation of catabolism genes. Recently, Krom *et al.* (2015) have shown superiority of phagemids, transferring antimicrobial peptides and bacterial toxins, compared to both lytic and lysogenic bacteriophage therapies, as they minimize the onset of resistance due to consistent expression and long-term stability of such constructs.

Delivery of antibiotic therapy supplements

Despite huge efforts in the development of new antibiotics, antibiotic-resistant bacterial strains still reappear, even when more types of antibiotics are used simultaneously. In addition, by horizontal gene transfer, the resistance quickly spreads among the bacteria. It has been shown that essential antibiotics such as gentamicin, if administered in great amounts, are toxic even for eukaryotic organisms (Ali 1995). The combination of phages and antibiotics in the treatment of bacterial infections could solve some of the drawbacks of antibiotics administration.

Yacoby *et al.* (2007) have investigated the possibility of targeted transport of antibiotic drug by a filamentous phage. Bacteriophage M13 was engineered to display fragments of polyclonal antibodies against the genera *Staphylococcus, Streptococcus* or against *E. coli* O78, respectively, fused to minor phage coat protein pIII, while the major coat protein pVIII was chemically modified via the linker by active chloramphenicol. Such system showed great decrease in the active drug amount necessary for a successful treatment. Another strategy for improving the antibiotic treatment has been used by Lu and Collins (2009). It was based on phage delivery of *lex*A3 encoding a repressor of bacterial SOS response, which plays an important role in the process of mutation induction and in the bacterial genome repair mechanisms (Fig. 2a). Knocking-down this response leads to a greater antibiotic-susceptibility of bacteria and reduction in both resistance development and its horizontal transfer (Beaber *et al.* 2004). The study analysed the synergistic effect of ofloxacin and the modified phage, revealing great efficacy both *in vitro* and *in vivo*. A similar concept of Libis *et al.* (2014) is based on RNA-mediated silencing of the genes responsible for antibiotic resistance, promoted by phagemid (Fig. 2b). Short regulatory RNAs (sRNAs) were designed to inhibit translation of chloramphenicol acetyltransferase and kanamycin phosphotransferase genes, inserted within the scaffold sequence of MicC, recruiting the degradation machinery promoted by the Hfq protein.

Phages were also engineered for dispersion on hospital surfaces to reduce the number of resistant pathogens residing in hospitals as well as the transfer of antibiotic resistance between strains. Edgar *et al.* (2012) constructed phage λ carrying the *rpsL* gene, mutant variants of which are responsible for the resistance to streptomycin, and *gyrA* that confers resistance to the quinolone drug family. The principle of this method is based on lysogenic conversion of the resistant strain after the application of phages carrying an antibiotic-susceptible allele of the gene that imparts resistance. Most recently, Yosef *et al.* (2015) have designed phage λ to deliver the CRISPR-Cas system,

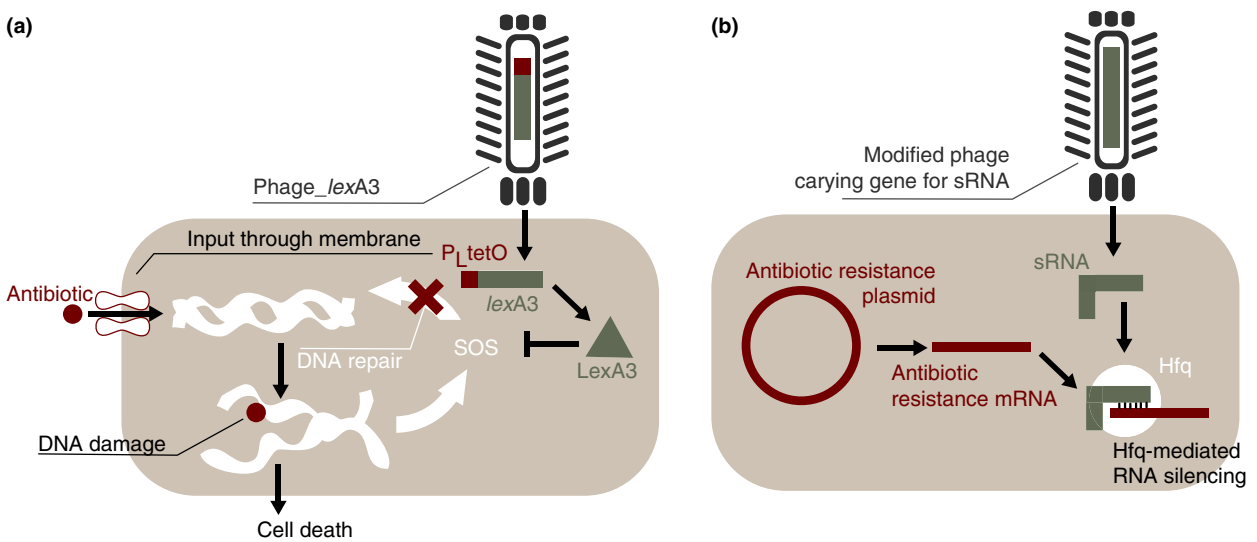

Figure 2 The strategies of phage-antibiotic synergy. (a) Antibiotic causes DNA damage, which cannot be repulsed by SOS response, due to the presence of over-expressed LexA3 protein, repressing SOS function. The gene *lex*A3 is placed under the control of $P_L$tetO promoter sequence, constitutively expressed in strains lacking tetracycline repressor. (b) RNA-silencing mediated by small regulatory RNA. sRNA is delivered to a pathogen by phagemid and binds to bacterial RNA via Hfq protein.

a prokaryotic system cleaving DNA sequences identical to particular segments, into bacteria to destroy DNAs that confer antibiotic resistance. By consequent incorporation of phage DNA into the bacterial genome, the bacteria render resistance to other related phages. After subsequent application of lytic phage T7, nonlysogenized pathogens are eliminated, and only CRISPR-Cas mediated antibiotic-susceptible cells remain.

Phage vaccine delivery

Phage display, as a method for identification of epitopes with high affinity to particular target, found its application in designing mimitopes of pathogen or tumour antigens, selected from the large phage libraries displaying random peptides (Menéndez *et al.* 2011). However, such peptides suffer from low immunogenicity and need adjuvants to elicit significant immune reaction (Skwarczynski and Toth 2016). Phage particles are processed by the immune system of mammals and can induce both cellular and humoral immune response (Ou *et al.* 2013; Tao *et al.* 2013). They are also resistant to the harsh physical and chemical conditions; therefore, they can serve as protective shells for vaccine delivery (Clark and March 2004). These properties together with cost-effective production and easy modification are opening doors for the new industry of phage-based vaccines fabrication, aimed at two separate approaches of antigen delivery: phage display vaccines and phage DNA vaccines. The former is based on the phage particle with antigen transcriptionally fused to its coat protein. The latter is based on the antigen gene, delivered by the phage into the antigen-presenting cell, where it is subsequently expressed and recognized.

Effective phage display vaccines have been developed for *Yersinia pestis* infections (Tao *et al.* 2013). T4 phage served as a delivery vehicle, displaying engineered structural subunit of *Y. pestis* on its capsid. Other vaccines based on pathogenic epitopes displayed on the phage surface were developed against HIV and influenza, utilizing phage T4 and T7 respectively (Sathaliyawala *et al.* 2006; Hashemi *et al.* 2012). Phagemid peptide display vaccines were developed against *Neisseria meningitidis* (Menéndez *et al.* 2011) and fungus *Candida albicans* (Wang *et al.* 2014).

Phage DNA vaccine against hepatitis B, utilizing engineered phage λ carrying viral surface epitope under the control of the cytomegalovirus expression cassette, served as a proof-of-concept of successful phage DNA vaccine delivery (Clark and March 2004). A similar approach was subsequently employed in the development of a vaccine against *Herpes simplex* virus 1, based on the engineering phagemid (Hashemi *et al.* 2010). Recently, a phage λ-based vaccine against *Chlamydia abortis* was designed, eliciting immune response superior to that elicited by the commercial vaccine Enzovax (Ou *et al.* 2013). Besides DNA vaccines, also an mRNA vaccine delivered by the phage MS2 was developed, showing promising results for future research (Sun *et al.* 2011).

Medical application of phage recombinant endolysins

For lysis of the bacterial cell, phages use a system based on two proteins, holin and endolysin, where holin mediates the disruption of the cytoplasmic membrane, thus enabling access of endolysin to peptidoglycan (PG), which causes cell degradation. In Gram-positive bacteria, a phenomenon called 'lysis from without' is observed when the cell is disrupted after the administration of endolysin from the outside. Therefore, endolysins have been considered for therapeutic use as enzybiotics and proven effective against a wide range of Gram-positive bacteria, offering several benefits (Schuch *et al.* 2002). The development of resistance to phage endolysins by the pathogens is unlikely due to their highly conserved target sites. Compared to phage particles, their pharmacokinetics and pharmacodynamics are easier to describe, which enhances their commercial potential.

Gene engineering is utilized in the preparation of recombinant endolysins, taking advantage of structural and functional independence of particular endolysin domains (Donovan *et al.* 2006). In general, phage lysins are composed of the cell wall-binding domain (CBD), responsible for affinity to the bacterial surface, and enzymatically active domain, rendering cleavage of PG. In addition, co-administration of multiple recombinant enzymes along with antibiotics reportedly results in a synergistic effect (Fernandes *et al.* 2012; Schmelcher *et al.* 2012).

Recombinant endolysins found use in the treatment of mastitis in dairy cattle, by constructing proteins effective against a wider range of pathogens causing mastitis (Donovan *et al.* 2006; Becker *et al.* 2009; Schmelcher *et al.* 2012). Besides the treatment of mastitis, recombinant endolysins were also developed and tested *in vivo* against eye (Singh *et al.* 2014) and skin infections (Pastagia *et al.* 2011) caused by *Staph. aureus,* without emergence of resistance. Another use of endolysins recombination was to increase solubilization and thus enhance purification of rather insoluble but lytic proteins, or their domains (Manoharadas *et al.* 2009; Fernandes *et al.* 2012).

The treatment of microbial infection by phage endolysins was not usable against Gram-negative bacteria due to the inability of lysins to cross the bacterial outer cytoplasmic membrane. However, Lukacik *et al.* (2012) were the first to construct a recombinant endolysin system effective against Gram-negative *Y. pestis*. This system is based

on a transport membrane protein, FuyA, one of the major virulence factors of the genus *Yersinia*. In the study, lysozyme of phage T4 was fused to pesticin, a ligand of FuyA (Fig. 3). After the administration of this recombinant lysin, only virulent *fuyA*+ strains showed significant reduction in viability. Moreover, the system is immune to the bacterial immunity protein Pim which inactivates natural pesticin. Another approach to crossing the outer membrane of Gram-negative bacteria was developed by Briers *et al.* (2014), who fused seven different peptides with endolysins to test their ability to destabilize the outer membrane and to lyse *Ps. aeruginosa*. The greatest antimicrobial effect was achieved with the polycationic nonapeptide (PCNP), showing both *in vitro* and *in vivo* efficacy in the reduction of *Ps. aeruginosa*.

Safety of phage products for medical application

Phages are completely natural and nontoxic for higher life forms than bacteria. However, they are out of classifications, as they are neither living nor chemical agents. Therefore, it is complicated to regulate their use and even have access to clinical trials and commercialization. Another problem represents the lack of acceptance by the general public due to a low awareness. It is supposed that the conventional approaches as GMP production are not compatible with a sustainable form of phage therapy (Pirnay *et al.* 2015). Serious safety concerns arise about medical application of phage products; therefore, minimal safety requirements for their release are the subject of scientific debate (Sybesma and Pirnay 2016). Environmental risk of phage-resistant bacterial strains emergence is not that severe, as the phenomenon of the phage resistance differs from antibiotic resistance due to evolutionary arms race in which phages and bacteria are locked (Pirnay *et al.* 2015). It often leads to loss of virulence as phages use structures responsible for virulence present on the bacterial surface as receptors (León and Bastías 2015). Joint efforts have been made to create a new European regulatory framework for phage therapy (Verbeken *et al.* 2014; Pirnay *et al.* 2015), driven by the international nonprofit organization Phages for Human Applications Group Europe (P.H.A.G.E.). The new framework would be helpful for ensuring the protection against newly arisen variants of pathogens, with the emphasis placed on treatment flexibility (Huys *et al.* 2013).

Further clinical trials with natural phages need to be conducted for an optimization of the regulatory guidelines for phage therapy (Vandenheuel *et al.* 2015). A good example in this regard is Phagoburn, an already running international project, assessing the safety, effectiveness, and pharmacodynamics of phage cocktails to treat burn wound infections. The major reason behind the low number of clinical trials of phages is that they are expensive, at least as expensive as those of antibiotics, and this along with the narrow host range of phages as well as the low predicted market price stemming from the hurdles for phage patentability as a natural product, reduce the interest of pharmaceutical companies in funding this research (Huys *et al.* 2013).

Genetically modified phages bring opportunity of intellectual property assignment, implying greater funding interest. Other problems of natural phages such as the content of virulence genes and ability of their transfer among bacteria also could be ruled out by artificial design (Nobrega *et al.* 2015). A major drawback of using modified phages seems to be the negative general view of people towards genetically modified organisms, although direct genetic modifications in principle do not differ from naturally occurring spontaneous mutations. Still, the environmental risk in the mean of an unpredictable effect of modified phages on the biosphere needs to be further investigated.

Biotechnology applications

Recently, bacteriophages have become a widely discussed issue in many fields of industrial application. With

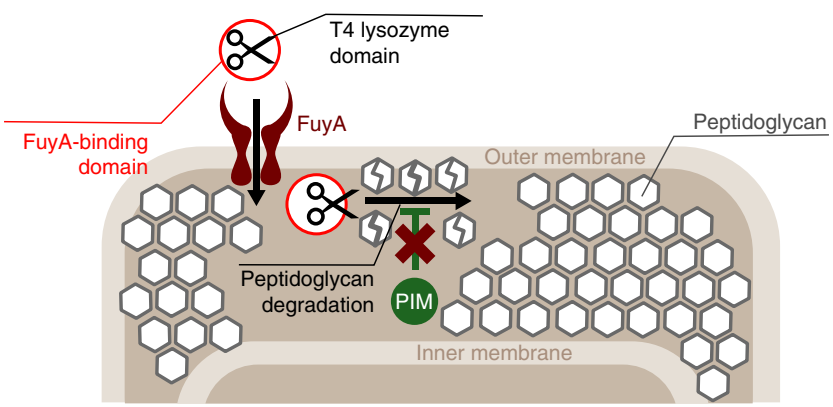

Figure 3 The strategy of endolysin application in the elimination of Gram-negative bacteria. The recombinant protein is capable of transport across the bacterial cell wall and mediates cell death. T4 lysozyme serves for the lysis of peptidoglycan, while the FuyA-binding domain mediates the transport of the whole protein through FuyA transporter via outer bacterial membrane. The immunity protein Pim is unable to inhibit the T4 lysozyme domain.

technologies such as phage display, they represent an easy and efficient way of protein and DNA vaccine engineering. The research of phage application also progresses rapidly in the food safety sector, in terms of both the detection and elimination of bacteria. Because of the lack of strict pharmaceutical regulation of their use in human medicine, phage-based products are more widely considered for use in the food industry. There are a number of approved products containing natural phages available on the market, for example, Listex™ P100 from Micreos Food Safety (Wageningen, The Netherlands), for controlling *Listeria* in meat, cheese and fish products (approved by the Organic Materials Review Institute, OMRI and European Food Safety Authority, EFSA), and the Intralytix products ListShield™ and SalmoFresh™ (approved by OMRI), all usable in food processing. Gene engineering plays a crucial role in the construction of phages capable of attaching to platforms, finding use in designing sensors or biosorbents for the elimination of food-borne pathogens. Besides, it opens the door for creating modified bacterial expression cultures capable of controlled autolysis, by incorporating phage lytic enzymes into the bacterial genome.

Biosensoring of pathogenic bacteria

Gastrointestinal epidemics, such as the one caused by shigatoxic strain *E. coli* O104:H4 in Germany in 2011, call for an urgent development of cheap and efficient tools for the detection of pathogens. To prevent such epidemics, the detection of possible contamination during transport of fresh products and also in each stage of the production process is crucial. The pathogen surveillance is even more urgent in the light of the concerns about the abuse of infectious agents by terrorists. The conventional microbiological methods for the identification are time-consuming, while molecular methods such as quantitative PCR (qPCR) or DNA hybridization rely on high purity specimens, struggling with the difficulty to discriminate between live and dead cells. Enzymatic assays such as ELISA show high sensitivity, but are not economically suitable for mass industrial applications. Usage of phages and phage-based sensors represents an alternative approach.

In the last decade, antibodies and bacteriophages have been considered for use as bioprobes in the detection of bacteria (Sorokulova *et al.* 2005), with phages possessing some significant benefits. Phages are possible to replicate in high titres at a low cost, they are more resistant to higher temperatures or pH fluctuations compared to antibodies and can be stored at room temperature with minimal loss of activity (Wan *et al.* 2007; Anany *et al.* 2011). Lytic phages were investigated as probes for bacterial diagnosis, but appeared to suffer from some serious drawbacks, as inability to bind to the detection matrix or narrow host range, which could be reverted by genetic modifications (Li *et al.* 2010; Javed *et al.* 2013).

Phages for specific delivery of reporter genes and fluorescently labelled phages

The strategy of transporting bioluminescent gene *lux* into *E. coli* via the reporter phage λ was introduced by Ulitzur and Kuhn (1989). After its expression in bacterial culture, the protein was quickly and sensitively detected by luminometer. Since then, several strategies based on transporting the *lux* gene, alkaline phosphatase or β-galactosidase gene for bacterial detection have been proposed and created (Table 1). One of the possible applications of these systems could be monitoring of antibiotic susceptibility of pathogens, as the luminescence signal response is dependent on host cell fitness (Alcaine *et al.* 2015).

Labelled phages are chemically or genetically modified by a detection element such as dye, nanoparticle or a protein, resulting in its covalent binding onto the phage coat surface. After creating phage with recombinant fluorescent coat proteins, it is possible to identify bacteria with several bound phage particles by epifluorescent microscopy. Awais *et al.* (2006) constructed GFP-labelled phage PP01 with defective lysozyme, specific to *E. coli* O157:H7. Such phage could propagate in bacteria, increasing the intensity of green fluorescence, but was unable to mediate host lysis, so there was no false drop in measured signal caused by bacterial lysis. Besides distinguishing dead from culturable cells, the proposed assay enabled the detection and quantification of viable but nonculturable cells, outperforming other conventional methods for pathogen detection.

To cope with the problems of low photo-stability and high signal-to-noise ratio of organic fluorophores, Edgar *et al.* (2006) in their study utilized quantum dots (QDs), the inorganic crystal semiconductors of nanometer sizes. They are characterized by strong photostability and high photoluminescence. In the study, they modified phage T7 by fusing its coat protein gp10A to a biotinylated probe 15 amino acids in size, which binds to QDs chemically modified by streptavidin. Subsequent refinement of this method produced a significant signal after the binding of only two phages on the bacteria, while phage particle alone produced a signal below the threshold (Yim *et al.* 2009).

Biosensors

Biosensors work on the principle of interaction of the analyte with a biological probe, transferred into

Table 1 Examples of phage-based pathogen detection systems

Type of sensor	Pathogen	Detection probe	Genetic modification of the probe	Assay duration	LOD (CFU ml^{-1})	Sample	Ref.
Reporter phage	*Escherichia coli*	Phage λ	::*lux* operon	100 min	10	Bacterial culture	Ulitzur and Kuhn (1989)
	Listeria monocytogenes	Phage A511	::*lux*AB	23 h	<1	Food	Loessner *et al.* (1996)
	Salmonella Typhimurium	Phage SPC23H	::*lux* operon	<2 h	10^2	Food	Kim *et al.* (2014)
	E. coli O157:H7 (VBNC)	Phage PP01	SOC/GFP	<12 h	Not stated	Bacterial culture	Awais *et al.* (2006)
	E. coli	Phage T4	::*β-gal*	5·5 h	<10	Water	Burnham *et al.* (2014)
	Pseudomonas cannabina	Phage PBSPCA	::*lux*AB	2 h	$1{\cdot}3 \times 10^3$	Bacterial culture	Schofield *et al.* (2012a)
	Bacillus anthracis	Phage Wβ	::*lux*AB	1 h	$7{\cdot}1 \times 10^2$	Blood	Schofield *et al.* (2013)
	Yersinia pestis	Phage φA1122	::*lux*AB	40 min	$6{\cdot}6 \times 10^2$	Bacterial culture	Schofield *et al.* (2012b)
	E. coli	Phage T7	::*pho*A	6 h	10^3	Bacterial culture	Alcaine *et al.* (2015)
Labelled phage	*E. coli*	Phage T7	Gp10A/biotinylated probe	1 h	20	Water	Edgar *et al.* (2012)
ME	*S.* Typhimurium	Phage E2*	pVIII/octapeptide	<1 h	10^2 to 10^3	Food	Park *et al.* (2013a,b)
	B. anthracis	Phage JRB7*	pVIII/octapeptide	1 h	5×10^2	Bacterial culture	Chai *et al.* (2013)
SPR	*L. monocytogenes*	Phage LmP4:A8*	pIII/scFv	Not stated	2×10^6	Bacterial culture	Nanduri *et al.* (2007)
	S. Typhimurium	Phage M13*	pIII/dodecapeptide	Not stated	$1{\cdot}3 \times 10^7$	Bacterial cocktail	Karoonuthaisiri *et al.* (2014)
	S. Typhimurium	RBP mP22	N-Cys/mP22	40 min	10^3	PBS	Singh *et al.* (2010)
	Campylobacter jejuni	RBP Gp047	Glutathione-S-transferase/Gp047	1 h	10^2	PBS	Singh *et al.* (2011)
	Bacillus cereus	CBD of LysPBC1	Glutathione-S-transferase/LysPBC1	1·5 h	10^3	Food	Kong *et al.* (2015)
Magnetic separation	*Camp. jejuni*	RBP Gp047	Glutathione-S-transferase/Gp047	<3 h	10^2	Food	Poshtiban *et al.* (2013)
	L. monocytogenes	CBD of Ply500	His tag/Ply500	6 h	10	Food	Kretzer *et al.* (2007)

/, genetic fusion; ::, insertion; CBD, cell wall-binding domain; CFU, colony forming units; GFP, green fluorescent protein; LOD, limit of detection; ME, magnetoelastic; PBS, phosphate-buffered saline; qPCR, quantitative PCR; RBP, receptor-binding protein; SOC, small outer capsid protein; SPR, surface plasmon resonance; VBNC, viable but nonculturable cells.
*Filamentous phage.

measurable signal by transducers. Magnetoelastic (ME) sensors and sensors based on the surface plasmon resonance (SPR) showed the best results in administration of the phage probes for the detection of the food contaminants. The ME sensor measures the change in the oscillation of the detection platform after exposure to an alternating magnetic field. As the pathogen binds to the phage immobilized on the platform, the mass of the platform increases which leads to a drop in platform resonant frequency. From this drop, the amount of the bound pathogen can be estimated (Fig. 4). This type of sensor is robust both in air and liquid and its quick response enables real-time and *in situ* detection of pathogens (Chai *et al.* 2013). As the overall frequency of the detection platform is measured, higher sensitivity can be achieved by minimizing the original mass of the sensor. ME resonator platforms are very cheap, which enhances their potential in the development of biosensors (Wan *et al.* 2007). As a probe, engineered filamentous phages had been studied (Sorokulova *et al.* 2005; Morton *et al.* 2013), displaying affinity peptide or antibody against pathogen fused to pVIII or pIII protein respectively, showing promising results (Table 1). For instance, phage E2, displaying on its pVIII octapeptide with specific affinity towards *Salmonella* Typhimurium, was attached to the ME platform of millimetre size, yielding maximal sensitivity of weight difference after *Salmonella* binding. Sensor showed to be competitive to currently used molecular methods in the estimation of the direct food contamination (Park *et al.* 2013a,b).

An SPR sensor evaluates the difference in light reflection, which depends on the sensor platform surface permittivity. When a pathogen is bound to a phage probe, immobilized onto the platform surface, a direct change in optical properties of the sensor occurs which can be subsequently measured and quantified. Genetically modified phages as well as their endolysin cell wall-binding domains and receptor-binding proteins (RBPs) responsible for mediating phage attachment to bacteria were evaluated as feasible probes for SPR sensors. As an example, RBP of bacteriophage NCTC 12673 was fused to glutathione-S-transferase, enabling its immobilization onto the surface of the sensor. Sensor could distinguish between *Campylobacter jejuni* and nonhost bacteria,

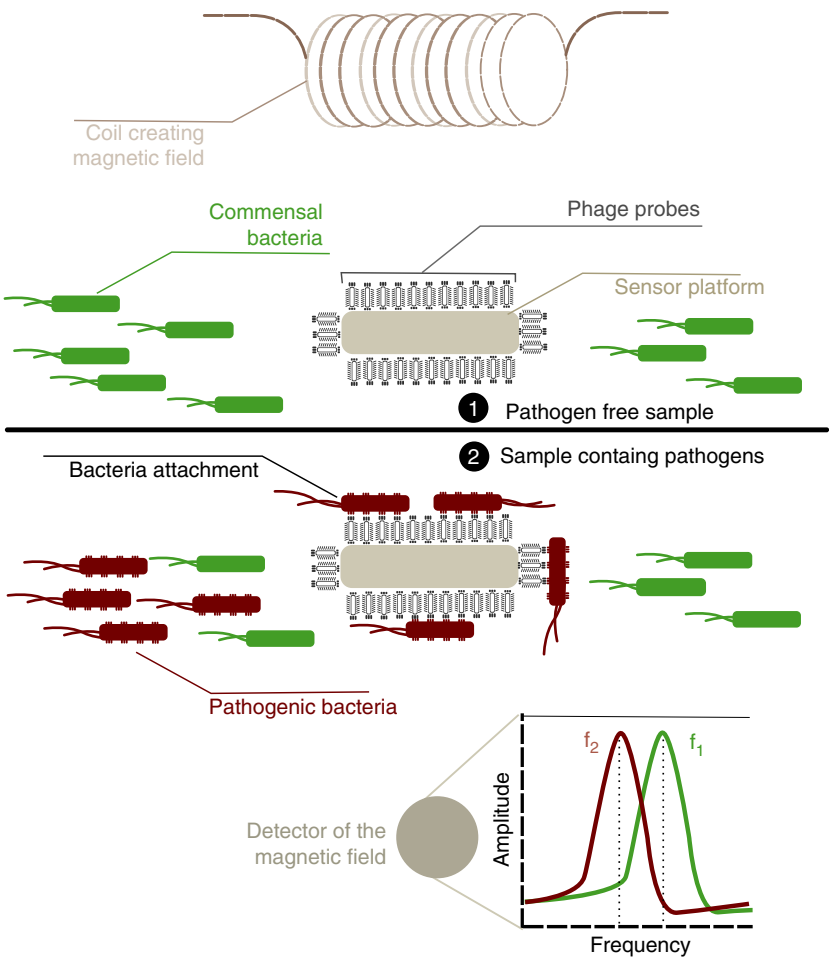

Figure 4 The scheme of a magnetoelastic sensor using a phage as a recognition probe. When the magnetic field is applied to the sensor platform, it oscillates with certain frequency. The phage, attached onto the platform serves as affinity probe and after the binding of the pathogen by its surface epitopes, a measurable decrease in resonance frequency from f_1 to f_2 will occur.

giving significant response after application as low as 10^2 cells (Singh *et al.* 2011). RBPs also found application as a probe for immunomagnetic separation in qPCR (Table 1).

Biosorbents

Phage-based materials applicable in the food industry for specific bacterial recognition have been increasingly investigated as they offer multiple advantages for mass industrial production. They are applicable in two practical fields: phage-based pathogen detection and deactivation of pathogenic bacteria. The key aspect is the immobilization of phages onto the surface of such a material. For this purpose, besides simple physical absorption, chemical and genetic modifications have been studied.

Phage T4 was engineered to display the biotin binding domain on the capsid head to allow a more efficient spatial arrangement of probes as phages were bound to the streptavidin-coated matrix only with the capsid head, so the receptor proteins on the tail remained free for binding the pathogen (Gervais *et al.* 2007). Another option of phage-based sorbent is the concept of cellulose biopaper. Due to its simple and cheap production and its great physical attributes, it is a promising candidate for use as a biosensor platform in industrial applications, suitable for a long-term storage (Anany *et al.* 2011). Li *et al.* (2010) displayed on the surface of phage T4 the cellulose-binding module of the thermostable xylanase from *Thermotoga maritima*. These phages have been proven to bind to cellulose with high affinity. To test the infectivity of the above-mentioned phage-based biosorbents, Minikh *et al.* (2010) evaluated growth inhibition of *E. coli* B after their administration. However, their study has shown the superiority of simple physical adsorption over modified phages, with both the biotin binding peptide and cellulose-binding module displaying phages struggling with low burst size and slightly prolonged phage latent period.

Recently, Chen *et al.* (2015) have compared the efficacy of magnetic nanoparticles functionalized with phages or antibodies, serving as bacteria-binding elements. They used phage T7 engineered to display a biotin tag on the coat, ensuring its binding to streptavidin-coated particles. Both systems have shown similar binding capacities and efficacy, with half the incubation time necessary for phage-functionalized probes. Such low-cost systems can be applied to separate specific bacteria from liquid samples.

Engineering phage lytic enzymes in biotechnology

Besides medical application, phage lytic enzymes show promising properties for utilization in industry fields. Recombinant endolysins were used in bacterial diagnosis for species like *Bacillus anthracis* (Schuch *et al.* 2002), *Bacillus cereus* (Kong *et al.* 2015) or *Listeria monocytogenes* (Kretzer *et al.* 2007; Schmelcher *et al.* 2010), applicable in food control as biosensor probes (Table 1). Besides the detection, recombinant endolysins could also be used as a preservation strategy in dairying (Schmelcher *et al.* 2012).

An interesting concept of endolysin engineering for biotechnology purposes is the cloning of phage lytic enzyme genes into vector systems or chromosomes to create bacterial systems capable of programmed cell lysis (Fig. 5). This concept finds its application in economically efficient secretion of cytoplasmic expressed proteins into culture medium, without any additional step of cell lysis leading to a loss of product. The proof-of-concept

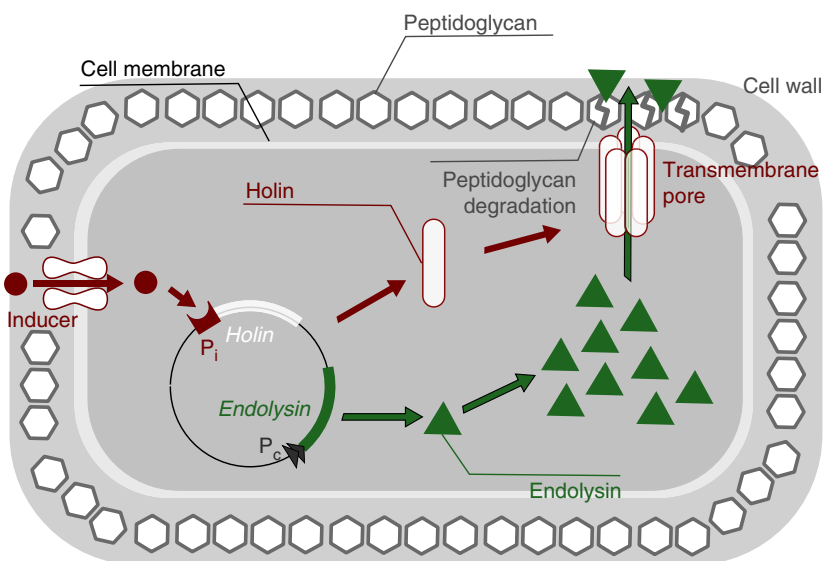

Figure 5 The principle of programmed cell death, mediated by phage lytic enzymes. The cell contains a plasmid encoding endolysin and holin genes, with the endolysin gene placed under a constitutive promoter, leading to accumulation of its product in the cytosol. The holin gene is placed under an inducible promoter. After addition of an inducer, holin is expressed and enables the endolysin transfer to peptidoglycan, which is consequently degraded and the cell is lysed even in the stationary growth phase.

was introduced using protein E of phage φX174 by Witte and Lubitz (1989), and subsequently applied in poly-β-hydroxybutyrate and polyhydroxyalkanoic acids granule secretion, usable as biodegradable plastic (Resch *et al.* 1998; Zhang *et al.* 2009). Autolysins were also employed in the biofuel production (Liu and Curtiss 2009; Miyake *et al.* 2014) or in the extraction of minicells (Park *et al.* 2011). Minicells are created due to improper assembly of the septum at cell division, with promising application as gene therapy vehicles.

The main disadvantages of the most aforementioned systems were the necessity of an additional inducer-adding step to mediate lysis. An alternative approach was taken by Yun *et al.* (2007) using T4 holin and T7 lysozyme placed under control of the *pts*G promoter inducible in the absence of glucose to create a vector that mediates recombinant protein release after glucose exhaustion in *E. coli*. Such a system can be applicable in high throughput screening of bacteria libraries for proteins with new desired properties, after directed protein evolution. In the large-scale industry, the major drawback of autolysis systems is the early emergence of mutants losing the inducible lysis phenotype (Yun *et al.* 2007; Liu and Curtiss 2009). Other systems independent from inducer-adding step were developed, controlled by heat shift (Cai *et al.* 2008) or light induction (Miyake *et al.* 2014).

Future prospects

The development of new techniques for phage engineering and their expansion to other phage types opens the door to a new era of phage application in both medicine and industry. Predictably, this potential will attract investment in phage research. Designing phages able to attack intracellular pathogens could be a crucial step towards solving the problem of drug resistant tuberculosis strains which are emerging as a serious threat worldwide. Identifying the mutations responsible for extending the host range of phages, larger burst size, or prolonged circulation time inside mammals can lead to the construction of more efficient phages both by genetic engineering and by conventional mutant selection. Not many studies so far have focused on such mutations; therefore, intensive research of phage genomics and proteomics is necessary for further development of phage-based products. Phage cocktails which overcome many obstacles in phage therapy when enriched by engineered constructs could be hugely efficacious; however, to our knowledge no study dealing with this issue has yet been published. Another open field in the medical application represents designing of a hybrid phage vaccine, functioning as both epitope display and DNA vaccine, which could be highly efficacious.

Genetically modified phages have some drawbacks like transfer of toxin genes by reporter phages. However, new strategies based on systems which are harmless to the environment, for example, phage or phagemid transferring antibiotic-susceptibility genes which do not mediate direct elimination of the bacteria, solve this problem. Still, the risk of recombination or horizontal gene transfer among phages and bacteria must be investigated, particularly for prophages or constructs with a wide host range. The future success of modified phages also depends on the health, safety and environmental regulation of genetically modified organisms in particular countries, so in the United States, there is a much greater potential for their early applications compared to the EU.

Acknowledgements

This paper was supported by grant MUNI/C/1501/2014 from the Masaryk University.

Conflict of Interest

No conflict of interest declared.

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