REVIEW

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Molecular Modification of T4 Bacteriophage Proteins and Its Potential Application – *Review*

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ABSTRACT. Bacteriophage T4 is a virus with well-known genetics, structure, and biology. Such techniques as X-ray crystallography, cryo-EM, and three-dimensional (3D) image reconstruction allowed describing its structure very precisely. The genome of this bacteriophage was completely sequenced, which opens the way for the use of many molecular techniques, such as site-specific mutagenesis, which was widely applied, *e.g.*, in investigating the functions of some essential T4 proteins. The phage-display method, which is commonly applied in bacteriophage modifications, was successfully used to display antigens (PorA protein, VP2 protein of vvIBDV, and antigens of anthrax and HIV) on T4's capsid platform. As first studies showed, the phage-display system as well as site-specific mutagenesis may also be used to modify interactions between phage particles and mammalian cells or to obtain phages infecting species other than the host bacteria. These may be used, among others, in the constantly developing bacteriophage therapy. All manipulations of this popular bacteriophage may enable the development of vaccine technology, phage therapy, and other branches of biological and medical science.

Abbreviations

Cryo-EM	cryo-electron microscopy
CSFV	classical swine fever virus
dsDNA	double stranded DNA
EF	edema factor
IgSF	immunoglobulin superfamily
LF	lethal factor
LPS	lipopolysaccharide
LTFs	long tail fibers
PA	protective antigen
PDPs	phage-display peptides
STFs	short tail fibers
vvIBDV	very virulent infectious bursal disease virus

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1 INTRODUCTION

Bacteriophage T4 is one of the largest viruses infecting bacteria. It is one of the best studied bacteriophages and a primary tool in molecular biology, mainly because of its simple handling and some technical advantages. First of all, it can be easily manipulated with classical genetics, including site-specific mutagenesis. It can also be easily grown in large quantities. T4 is one of the most complex viruses, but its structure was investigated and very well described using X-ray crystallography, cryo-EM, and three-dimensional (3D) image reconstruction (Fokine *et al.* 2004; Mesyanzhinov *et al.* 2004; Olson *et al*. 2001).

The bacterial strains infected by T4 are *Escherichia coli* and the closely related *Shigella*. This bacteriophage is a member of the *Myoviridae* family. It consists of a prolate icosahedral head, a collar with whiskers, a contractile tail, and tail fibers. The fibers are engaged in the recognition of the host cell surface and attachment of the phage to the bacterium during the infection process. The tail ends with a baseplate which binds to the specific receptors on the host's surface. It is also equipped with enzymes able to degrade the bacterial wall, which enables introducing nucleic acid into the cell. The tail works as a syringe. During its contraction, the nucleic acid is pushed into the bacterial cell. The genome, which is dsDNA, is tightly packed in the protein capsid. Considering that some viruses have genomes containing only 5×10^3 bp (bac-

teriophage φX174), T4 seems to be a giant (Mesyanzhinov *et al*. 2004). Its DNA consists of 1.69×10^5 bp constituting 289 open reading frames, eight tRNA genes, and at least two genes whose products are small RNAs of unknown function. More than 150 genes of this virus have been characterized, 62 of which have been described as "essential". The virion consists of about 50 different kinds of proteins (Mesyanzhinov *et al*. 2004), of which at least 12 build the T4 head (Leiman *et al*. 2003). Except for nucleic acid, there are only a few non-protein constituents in the virion: polyamines associated with DNA (putrescine, spermidine, cadaverine), ATP and Ca^{2+} associated with the tail sheath, and dihydropteroylhexaglutamate associated with the baseplate (Birge 2006). Nowadays, knowing the structure of bacteriophage T4 quite well and also having a broad range of genetic techniques as a molecular tool, we can change the properties of this virus almost any way we want. Therefore, this virus has the chance to be a kind of biotechnological "guinea pig", and its modifications may open new avenues for molecular and medical biology.

2 THE PROTEIN STRUCTURE OF T4 HEAD

The head of bacteriophage T4 has a relative molar mass of 194×10^6 g/mol and is 115 nm long and 85 nm wide. The head is an icosahedron consisting of 160 hexamers of gp23 (major capsid protein, 48.4 kg/mol), 11 pentamers of gp24 (pentameric corner protein, 46 kg/mol), and 1 dodecamer of gp20 (Table I). During the formation of the prohead, scaffold and shell proteins undergo proteolytic cleavage by

Table I. The protein structure of the head of bacteriophage T4[a]

Gene name	Mass kg/mol	Size (number of amino-acid residues)	Number of copies in mature head	Location
20	61.0	524	12	shell, portal
21	18.5	♦	♦	internal core, proteinase
22	2.5	♦	♦	internal core, major protein
23	48.7	422	930	shell, major capsid protein
24	46.0	407	55	shell, vertices
67	3.9	♦	♦	internal core
68	15.7	♦	♦	*ditto*
Alt	75.9	682	40	*ditto*
Hoc	39.1	376	155	shell, outer surface
IPI	8.5	♦	♦	internal core
IPII	9.9	♦	♦	*ditto*
IPIII	20.4	♦	♦	*ditto*
Soc	9.7	80	810	shell, outer surface

[a]Modified from Fokine *et al.* (2004) and Leimann *et al*. (2003).

♦ – proteins forming a scaffold, coated with gp23 and gp24 during the maturation process. After prohead assembly, the scaffolding core structure is proteolytically removed and the core proteins are either totally or partially degraded. The degradation products of some core proteins remain inside the mature head. Only the internal proteins (IPI, IPII, and IPIII) are found in comparable amounts in the prohead and in their processed form (Kuhn *et al*. 1986).

the active form of gp21 (prohead proteinase, T4PPase). The amino termini of proteins gp23, gp24, IPI, IPII, IPIII, and gpalt are cleaved, while proteins gp22, gp21, gp67, and gp68 are extensively digested (Leiman *et al*. 2003). The distance between the gp23 hexamer centers is ≈14 nm (Fokine *et al*. 2004). These two proteins form a ≈3-nm-thick shell protecting the nucleic acid. Head corners are occupied by gp24, which forms pentamers and interacts with the borders of the gp23 hexamers (Fig. 1). Another structural element of the T4 capsid is a small protein, Soc (small outer capsid protein, 9 kg/mol). It forms an almost continuous mesh on the surface of the gp23 hexamers. It binds two gp23 subunits, but it cannot bind around the gp24 pentamers or between gp23 and gp24. Some studies also revealed that Soc–gp23 interactions are favored over Soc–Soc interactions (Olson *et al*. 2001). The lattice created by gpSoc encircles each gp23 hexamer which does not border on a gp24 pentamer. When gp23 is neighboring with gp24, Soc molecules occupy only five of the six sides of the hexagon. It is not incorporated into the side which is attached to the gp24 pentamer. Most of the gp23 molecules are in contact with two Soc proteins, except for those which are closest to a gp24, which interact with only one Soc particle. The location of the Soc molecules seems to confirm one of their proposed functions, which is reinforcing the vaults between gp23 subunits. When located between gp23 subunits,

gp Soc forms trimers, but when purified from the phage or expressed *in vitro* it has the form of a monomer. Although the function of Soc protein has not yet been fully characterized, it is considered to stabilize the phage's capsid against thermal denaturation and exposure to detergent or alkaline pH, and is thus responsible for preserving phage viability in unfavorable conditions (Olson *et al*. 2001).

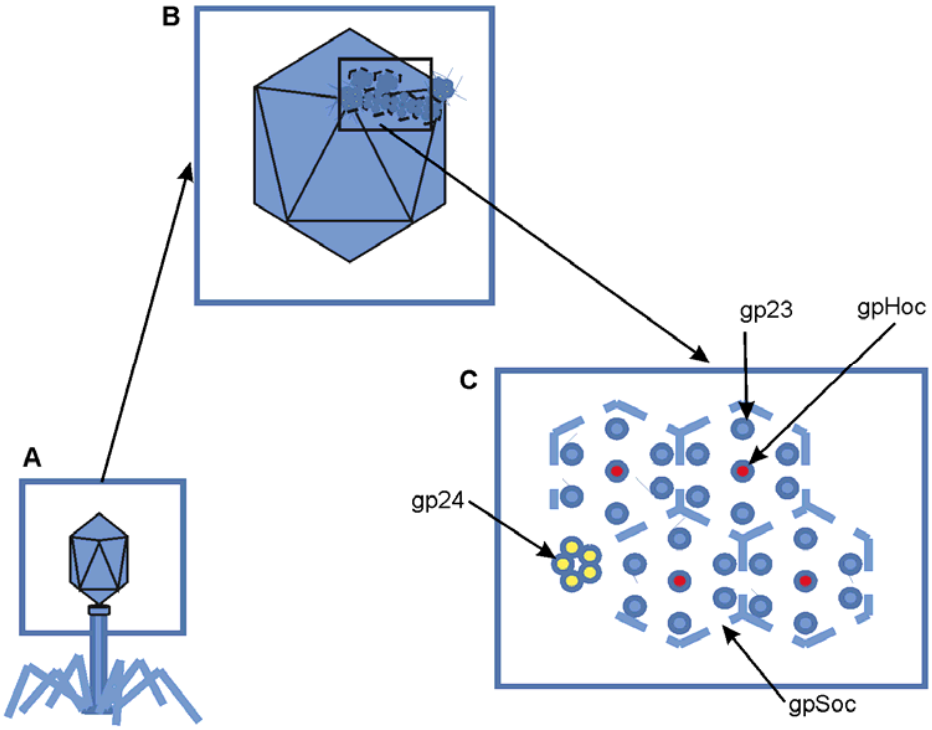

Fig 1. Protein structure of bacteriophage T4 head. **A**: General structure of bacteriophage T4 virion. **B**: Bacteriophage T4 head. Arrangement of proteins on head surface. Pentamers of gp24 are placed on head's corners while the rest of the surface is occupied by units made of gp23, gpSoc and gpHoc. **C**: Reciprocal arrangement of head proteins. Hexamers of gp23 are surrounded by gpSoc except borders with gp24 pentamers. The middle of each hexamer is occupied by gpHoc.

gpHoc (Highly Immunogenic Outer Capsid Protein, 39.1 kg/mol) is probably the most characteristic protein of the T4 capsid because of its regular location in the middle of each gp23 hexagon and because it protrudes considerably from the surface of the phage's head. This protrusion extends ≈5 nm over the capsid shell and it has a molar mass of ≈12 kg/mol. Hoc protein has a multidomain structure. It consists of a ≈0.19-nm-high round base, a thin neck region, and a ≈2-nm-wide and 2.4-nm-high globular head. The function of gpHoc is unknown (Leiman *et al*. 2003).

3 THE PROTEIN STRUCTURE OF THE TAIL AND FIBERS

The T4 tail and fibers are important tools for bacteriophage interactions with its host. This part of the capsid determines phage specificity and enables the infecting of bacteria. The tail is composed of two concentric protein cylinders. The outer cylinder is contractile and the inner one builds the channel for the nucleic acid that is stored in the head. Such a syringe-like construction of the tail enables the injection of DNA into the bacterial cell. The inner tube has a 9-nm outer and 4-nm inner diameter and is constructed of 144 copies of gp19 (Table II). The outer part of the tail is called the tail sheath. It is built by 144 copies of gp18 (in accordance with the number of gp19). The length of the tail is probably determined by the "ruler protein" (or template) gp29 (Leiman *et al*. 2003). The uncontracted tail is 100 nm long and 21 nm in diameter (Mesyanshinov *et al*. 2004), which corresponds to the dimensions of the tail sheath. When contracted, the tail sheath is only 36 nm long and 27 nm wide. The length of the tail tube does not change during contraction (Leiman *et al*. 2003). At either end of the cylinders are the baseplate and fibers. The baseplate is a multiprotein structure 27 nm high and 52 nm in diameter in its widest part. The proteins form six wedges surrounding the central hub with the help of two trimeric proteins, $(gp9)_3$ and $(gp12)_3$. gp11, gp10, gp7, gp8,

gp6, gp53, and gp25 combine sequentially to build up the wedges. gp5, gp27, gp29, and probably gp26 and gp28 form the baseplate's hub (Leiman *et al*. 2003). gp5 has a lysozyme domain which is necessary for the digestion of the bacterial peptidoglycan layer during the infection process.

Table II. The protein structure of the T4 phage tail and fibers[a]

Gene name	Mass kg/mol	Size (number of amino-acid residues)	Number of copies	Location
3	19.7	176	6	tail tube terminator
5	63.7	575	3	central hub
6	74.4	660	12	baseplate wedge
7	119.2	1032	6	*ditto*
8	38.0	334	12	*ditto*
9	31.0	288	18	wedge – vertex
10	66.2	602	18	wedge – pin
11	23.7	219	18	*ditto*
12	55.3	527	3[b]	baseplate
15	31.4	272	6	tail terminator
18	71.2	659	144	tail sheath
19	18.5	163	144	tail tube
25	15.1	132	6	baseplate wedge
26	23.4	208	nd	chaperone
27	44.4	208	3	central hub
28	24.0	177	nd	*ditto*
29	64.4	391	6	tail tube
34	140.0	1289	3[b]	proximal part, connected to the baseplate
35	30.0	372	1[b]	hinge region
36	23.0	221	3[b]	distal part, hinge connection
37	109.0	1026	3[b]	distal part, receptor recognition tip
48	39.7	177	6	baseplate wedge
53	23.0	196	6	*ditto*
54	35.0	590	6	*ditto*
Frd	21.7	320	6	baseplate wedge?
Td	33.1	364	3	central hub ?
wac	51.9	89	3[b]	head-tail joining region

[a]Modified from Mesyanzhinov *et al*. (2004) and Leimann *et al*. (2003); nd – not determined.
[b]The number of copies of fiber proteins is given per fiber. There are six fibers of each type per T4 virion.

One of bacteriophage T4's most useful devices are the fibers: LTFs and STFs located on the distal part of the tail and whiskers extending outwardly from the collar region of the virion (Conley and Wood 1975). The long tail fibers, which are responsible for the recognition of specific receptors on the bacterial surface, are ≈145 nm long and ≈4 nm in diameter (Leiman *et al*. 2003). Each fiber consists of two halves: the proximal half encoded by gene *34* and the distal one encoded by genes *36* and *37*. These halves are connected by gp35, which interacts with gp34 and gp36. The protein connecting the LTFs with the baseplate is gp9. Association of the proximal part of the fiber with gp9 is assisted by gp63. gp9 has an extremely important role during infection. After the binding of an LTF to the LPS on the bacterium wall, it initiates the transition of the baseplate structure into a star-like conformation and the tail sheath contraction that enables injection of the phage's DNA into the cell. Besides that, gp9 is also responsible for the collective movements of the fibers and for preventing the baseplate from abortive triggering. The short tail fibers are gp12 trimers attached to the baseplate by gp11. They are 34-nm-long club-like structures with a narrowing in the middle where the fiber can bend up to 90º. STFs are responsible for binding the phage particle to the bacterial surface. During the infection process the C-termini of gp12 molecule bind to the core region of the LPS cell-surface receptor (Mesyanzhinov *et al*. 2004).

4 MUTAGENESIS IN T4 BACTERIOPHAGE GENOME

Bacteriophage T4 and other T-even phages were used as molecular models in early studies of induced mutagenesis. The results of these studies were often adapted to higher organisms because of the similar

mechanisms of mutagenesis. Spontaneous mutations (usually point type) occur in the T4 genome with a frequency of about 10^{-7} per bp in one replication cycle. However, a much higher mutation rate can be induced by many physical and chemical agents, *e.g.*, UV irradiation (bacteriophage T4 can be inactivated at a wavelength of 253.7 nm), heat (Kricker and Drake 1990), and some chemical agents: the base analogues (2-aminopurine, 2,6-diaminopurine and 5-bromouracil), hydroxylamine, ethyl methanesulfonate, nitrous acid, and others (Switała-Jeleń *et al*. 2002).

It is also possible to use site-specific mutagenesis, which allows introducing a mutation in selected genes. The complete T4 genome has now been sequenced (Ackermann and Krisch 1997) and there is a vector-based system allowing the insertion of *in vitro* generated mutations into the phage genome (Selick *et al*. 1988). The system was used to investigate the functions and structures of many important gene products, for example T4 lysozyme, DNA polymerase, endonuclease V, and regA protein. The application of systematic site-directed mutagenesis allowed pointing out protein sites sensitive to amino-acid substitution. These sensitive sites contain the key amino acids of the protein (Rennell *et al*. 1991). This method made it possible to investigate the roles of individual enzyme fragments and confirm previously developed hypotheses describing their function and structure (*e.g.*, T4 lysozyme). Site-specific mutagenesis was also used to clarify the role and interactions between proteins involved in the synthesis of DNA by T4 DNA polymerase (Karam and Konigsberg 2000; Kunkel *et al*. 1984) and helped to define the function of the T4 phage protein regA (a repressor regulating the translation of at least 12 genes by binding to mRNA) (O'Maley *et al*. 1995). Site-directed mutagenesis of the N-terminus of endonuclease V was used for investigation of the parameters influencing cleavage mechanisms. The studies indicated the importance of the distance between the α-NH_2 group at the active site of the enzyme and the sites responsible for DNA binding and recognition of pyrimidine dimers (Schrock and Loyd 1993).

Because of the ready creation of collections of different "mutant proteins" and their comparison with "normal" ones, site-specific mutagenesis is a valuable method for investigating protein function and structure. This knowledge may then lead to further engineering of their properties.

5 PHAGE-DISPLAY SYSTEM

One of the most advanced and most popular genetic methods used to modify the properties of bacteriophage T4 (and other phages) is the phage-display system. It is based on the idea of the fusion of genes encoding phage and "non-phage" proteins and their expression on the bacteriophage surface. The product of such fusion is a foreign protein built into the phage virion. The key moment in the development of this method was the work of Smith (1985), who fused the f1 bacteriophage capsid protein pIII and bacterial endonuclease *Eco*RI. The sequence that encodes the endonuclease was introduced into the f1 phage genome. The protein was incorporated into the virion particle; moreover, the phage partially kept its infectivity and original immunogenicity. In further studies, the region of cloning was moved to a sequence encoding the N-terminus of pIII, which resulted in obtaining the phage with full infectivity. Since then, different phages (*e.g.*, M13, λ, T4) have been used to obtain PDPs, *i.e*. proteins exposed on the phage surface (Borysowski and Gorski 2004). The phage-display technique has been applied in many laboratories and appears to be a potent tool for selecting specific molecules. Displaying a variety of proteins (*e.g.*, antibodies) on numerous individual phage particles enables the creation of a collection of various molecules on a phage vector pool. Such collections are called phage libraries. They are used mainly for defining protein ligands for different molecules. Such a library allows easy selection, which includes three steps: (*i*) incubating the molecule with the phage library, (*ii*) removing unbound phage particles, and (*iii*) amplifying the selected phages in *E. coli* cells (Paschke 2006). The phage-display technique has been used for many different purposes: identifying protein–ligand interactions, including screening for receptor agonists and antagonists, defining epitopes of monoclonal antibodies, selecting enzyme substrates or inhibitors, designing and improving enzyme properties, selecting targets for the inhibition of tumor-specific angiogenesis, combating infectious diseases, identifying peptide drug candidates, and for vaccine development (*e.g.*, vvIBDV, CSFV, anthrax toxin, HIV, most of these discussed *below*) (Benhar 2001; Fernandez-Gacio *et al*. 2003; Konthur 2002; Mullen *et al*. 2006; Sergeeva *et al*. 2006; Willats 2002; Wu *et al*. 2006). This method has several advantages: the very high number of proteins that can be displayed, high flexibility, the selection may be performed *in vitro* and *in vivo*, *in vitro* selection may also involve inorganic molecules, and the technique is effective, fast, cheap, easy to control, and requires no special equipment (Willats 2002).

T4 has some features which favor this virus in studies aimed at displaying protein molecules on the capsid surface, especially for creating multicomponent vaccines. Bacteriophage T4 has two inessential proteins, Hoc and Soc, located symmetrically on the capsid surface. Modification or removing these by muta-

tion does not affect the phage's productivity, viability, or infectivity (Jijang *et al*. 1997). These proteins are displayed at high copy numbers and are added to the T4 head after completion of capsid assembly but before DNA packaging. The high copy number of Hoc and Soc enables displaying more than one fusion protein on the same capsid and also allows controlling their number. The number of copies can be regulated by changing the binding conditions (Shivachandra *et al*. 2006). Many proteins of different size, structure, and biological function have been successfully fused to the T4 capsid. Recent studies focus on attempts to display antigens of some pathogens, which could potentially lead to the development of very functional vaccines. Bacteriophage T4, as described *below*, was used not only as a display surface for relatively large proteins but also for displaying more than one different protein in various combinations at the same time. The fact that the capsids can be devoid of DNA (displaying peptides on DNA-free capsids is possible) and have numerous tail proteins is extremely important for the use of PDPs as vaccines. The lack of DNA prevents uncontrolled gene transfer or antibacterial activity. The tail proteins interact with the immune system. These interactions may be desirable when the tail components interfere in a negative way with the immune response elicited towards the displayed proteins, but they are undesirable when the tail proteins offer favorable adjuvant effects (Jijang *et al*. 1997).

6 T4 CAPSID AS A PLATFORM FOR THERAPEUTIC AGENTS

Jijang *et al.* (1997) were among the first investigators to study the potential use of bacteriophage T4 as a platform for vaccines. These studies showed that the features of this virus allow displaying peptides not only on complete virions, but also on empty capsids. The 36-amino-acid PorA peptide of *Neisseria meningitidis* was cloned into the display vectors to generate fusions at the N-termini of Hoc and Soc. Stable peptides incorporated into the T4 capsid in an accessible form were obtained. Experiments *in vivo* confirmed the immunogenic properties of the displayed PorA. These studies showed that the T4-based system enables regulation of the number of copies of displayed peptides as well as the size and the structure of the virion in order to induce optimal immune response. These properties make T4 bacteriophage a very useful tool for creating advanced vaccine strategies (Jijang *et al*. 1997).

Bacteriophage T4 was used to obtain a vaccine against infectious bursal disease in chickens caused by vvIBDV (very virulent infectious bursal disease virus). The major immunogenic protein VP2 of the vvIBDV was fused with Soc protein on the T4 capsid surface. The system was based on recombination of plasmid with VP2 cDNA fused to the *soc* gene and the nucleic acid of T4-Z1, a mutant phage with deleted *soc* gene. The antigenic site responsible for the induction of neutralizing antibodies is highly conformation dependent. Although information concerning the configuration of the displayed and native particles is lacking, there are grounds for accepting that the display of VP2 on the T4 surface enabled maintaining the conformation of the epitopes so that a high humoral response in chickens was elicited. Furthermore, birds previously immunized with T4-VP2 did not reveal any clinical signs of disease after infection with vvIBDV. In this group of chicken, no deaths were observed. Interestingly, some damage caused by bursal disease was noted, but it seemed to have been spontaneously repaired shortly after infection. Considering the effectiveness of the applied technique and the inexpensive production, convenient storage, and transport of potential phage vaccines, this method seems to be an efficient way to prevent IBD in chickens. These studies were very important also because of the size of the fusing protein. The attempt to display a peptide of 441 amino acids on the bacteriophage's surface was probably one of the first attempts to display such a large protein. The fact that the recombinant mutants were immunogenic, antigenic, and effective in protecting birds from IBD proves that this technique is elastic and may even be used to display large proteins (Yong-Chang *et al*. 2005).

These observations were confirmed in studies of binding anthrax-protective antigen (PA) to the T4 bacteriophage capsid. The system applied was based on specific Hoc–capsid interactions allowing the binding of a PA–Hoc fusion to the T4 *hoc*$^{-}$ mutant's capsid in an *in vitro* reaction. The studies indicated no significant limitations on the size of the displayed antigen (Shivachandra *et al*. 2005). Later, the same system was used to create a multicomponent anthrax vaccine. The anthrax-toxin protein-PA, lethal factor (LF), and edema factor (EF) were fused to the N-terminus of Hoc and displayed on the T4 surface either individually or in combinations. The displayed antigens elicited a strong immune response in mice (interestingly, the presence of the LF and EF antigens reinforced the PA-specific immune response compared with the PA displayed individually) with the absence of an externally added adjuvant. The studies showed a possible way to obtain multicomponent vaccines, which is extremely important because pathogens usually encode more than one antigenic region and the majority of vaccines target the response of only one antigen (Shivachandra *et al*. 2006).

Very promising were also the results of studies on creating a multicomponent HIV vaccine. Three HIV antigens were displayed on the T4 surface: p24, Nef, and g41. p24 was chosen as a model antigen to develop the system and to determine the immunogenicity of the fused protein. Declining sera titers of p24 antibodies are connected with poor patient condition and progression of the infection to AIDS. Nef, the second displayed antigen, exerts multiple effects by interfering with cellular gene expression and signaling pathways which also result in the progression of HIV to AIDS. The last antigen was an engineered gp41 C-peptide trimer, also a valuable target for vaccine design because of its exposure during the fusion of the HIV envelope to the host cell. The antigens were fused to Hoc protein in expression vectors, expressed in *E. coli* cells, purified, and displayed *in vitro* on T4 *hoc*$^{-}$ mutants. Single-, double-, and triple-antigen-displaying capsids were obtained. The displayed p24 protein was highly immunogenic and elicited strong antibody and cellular response with very small amounts of bound antigen. Prior to studies on using T4 bacteriophage capsid, some efforts had been made to obtain such vaccines (*e.g*., using mixtures of proteins); however, all these systems were characterized by limitations such as the cost and the complexity of production. *In vivo* methods face such problems as the low and variable copy number of the displayed antigen, unspecific proteolysis leading to the loss of critical epitopes, and structural heterogeneity due to aggregation, insolubility, and improper folding of proteins. These problems may be overcome by *in vitro* techniques (Sathaliyawala *et al*. 2006). It is obvious that many studies on the application of phages as platforms for vaccines still need to be done; however, considering the early research results and the elasticity and simplicity of the system, it may be considered an attractive alternative to other solutions. Presented data are summarized in Table III.

Table III. Antigens displayed on bacteriophage T4 capsid

Phage protein used for antigen display	Protein displayed on phage capsid	Native occurrence of antigen
Soc	VP2	vvIBDV
Hoc	PA, LF, EF	anthrax toxin
	p24, Nef, gp41	HIV virus
Soc and Hoc	PorA	*Neisseria meningitidis*

7 POTENTIAL CONSEQUENCES OF T4 MUTATIONS FOR PHAGE THERAPY

One of the objectives of bacteriophage engineering is developing and streamlining of bacteriophage therapies. Phage therapy is an alternative to antibiotic treatment. The therapeutic application of bacteriophages has, however, a few main limitations, such the high specificity of bacteriophages (even generating problems in preparing active strains) and the removal of phage particles from the organism as a result of immunological barriers and the patient's immune response (Kutter 1997). These specific problems of antibacterial bacteriophage treatment may be resolved by relatively slight modifications of the bacteriophage capsid structure. Merril *et al.* (1995) selected mutants of a derivative of the λ phage and a derivative of P22 phage. The mutants not only remained in the murine circulatory system for a longer period of time, but were also more effective as antibacterial agents than the parental strains. These special properties resulted from a single point mutation in the major capsid protein of the λ-like phage: E protein (P22 derivatives were not analyzed). The mutation comprised a $G_{6606} \rightarrow A$ transition that resulted in the change of glutamic acid (158) for lysine (Merril *et al*. 1995). Although there are no similar data related to T4, this interesting report shows the possibility of designing phage response to clearing and provides a new idea for constructing other long-circulating phages, including T4.

Interestingly, a "short-circulating" mutant of bacteriophage T4 was constructed. The mutant, HAP1, was selected *in vitro* as a substrain of T4 that strongly interacts with the mammalian cell line. Surprisingly, *in vivo* tests showed a much more intensive clearing of this phage from the murine organism. This mutant bears a non-sense type mutation in the *hoc* gene and consequently lacks gpHoc at its capsid. This probably causes exposure of other active and immunogenic elements of the T4 capsid and invites an intensive reaction of the organism (Dąbrowska *et al*. 2007). These observations show that engineering bacteriophage susceptibility for removal (in phage therapy) can be very elastic and comprehensive. According to the specific clinical needs, the circulation of the phage may possibly be prolonged or reduced.

The HAP1 mutant also reveals other properties that differentiate it from its parental strain T4 and that can possibly be used in regulating the immunological effects of bacteriophage treatment. It was shown

to interact significantly more weakly than T4 with platelets (Kniotek *et al*. 2004). On the other hand, its affinity for cancer cells was significantly stronger (which was the principle of selection of this mutant). HAP1 preparation was also able to exert some substantial inhibitory effect on experimental tumor development in mice (Dąbrowska 2004*a*,*b*).

One problem in phage therapy is the high specificity of bacteriophages to bacterial strains. The specificity of T4 is determined by the end of the gene *37* encoding the tail protein, which binds to the bacterial receptors. Controlled mutagenesis makes it possible to obtain viruses with altered specificity. Bacteriophage T4 infects *E. coli* bacteria and the closely related *Shigella* sp. However, there are some mutants able to adsorb on cells of *Yersinia pseudotuberculosis*, bacteria which are evolutionarily distant from *E. coli*. One type of these mutants has point mutations in the C-terminal fragment of gene *37* which causes the substitution of one or two amino acids in this section. The second type of mutation involves unequal exchanges between a series of sequence motifs (His boxes) in the same region. Duplication or mutational changes in this fragment cause adsorption of bacteriophage T4 to *Y. pseudotuberculosis* receptor. It has been suggested that recombination of the His box sequences may result in changes in bacteriophage specificity as a consequence of modifications in the proteins responsible for phage binding. We may thus obtain phage mutants with induced ability to destroy new bacterial strains (Tetart *et al*. 1996).

The T2 bacteriophage is very closely related to T4, with high genomic homology and structural similarities. In studies by Yoichi *et al*. (2005), genes encoding the T2 tail fiber proteins gp38 and gp37 were exchanged with those of PP01 phage as a result of homologous recombination of the T2 phage genome and a plasmid encoding the region around genes *37–38* of PP01. The mutant T2 phage could infect the heterogeneous host *E. coli* O157:H7 and related species. Interestingly, it could not interact with its natural host, *E. coli* K12, and its derivatives (Yoichi *et al*. 2005). Other T4-like phages, TuIa and TuIb, and T4 itself were investigated by Hashemolhosseini *et al*. (1994), who demonstrated the role of protein 38 in determining phage specificity and phage sensitivity to "non-permissive" temperature. As expected, some changes in these properties resulted from substitution mutations of amino acids in the variable region of dimeric gp37, but also in protein 38. This protein is not a component of the phage capsid, but it is required for gp37 dimerization. Therefore, gp38 was classified as a molecular chaperone and proposed to act instructively in conveying steric information to the target polypeptide (Hashemolhosseini *et al*. 1994).

Some advanced experiments on modifying phage specificity show the possibility of changing it dramatically by the phage-display technique. Di Giovine *et al.* (2001) introduced a gene of the adenovirus penton base protein into the genome of bacteriophage M13. This protein is responsible for binding and internalizing adenovirus into the host cell. It is commonly known that bacteriophages are viruses which have no natural tropism for eukaryotic cells. M13 bacteriophages displaying the full-length penton base protein or its 107-amino-acid central region could bind, internalize, and transduce mammalian cells expressing integrin receptors. Both engineered phages bound $\alpha v\beta_3$, $\alpha v\beta_5$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ integrin subtypes. Although such a modified bacteriophage could interact with mammalian cells, it was not able to propagate inside them and did not cause cells lysis (Di Giovine *et al*. 2001). Other studies showed that expression of *Helicobacter pylori*-binding peptide on the same phage (M13) resulted in the effective killing of *H. pylori* by the engineered phage *in vivo* (Cao *et al*. 2000). These studies concerned a virus which is quite distant from T4; however, they demonstrate a very inspiring and potent idea for designing phage specificity with common biotechnology tools such as the phage-display technique. This idea opens new ways for studies on T4 phage.

8 THE PROSPECTS OF T4 BACTERIOPHAGE MOLECULAR MODIFICATIONS

The postulated role and significance of bacteriophages has increased dramatically in recent years. We are starting to appreciate the role of naturally existing bacteriophages: they are ubiquitous and extremely numerous. One can find them in animal and human bodies and in the environment (water, soil). Bacteriophages constitute a considerable part of the human microflora and should be regarded as important factors influencing our health and immunological status (Dabrowska *et al*. 2005). Bacteriophage T4 is one of the most popular representatives of the "phage family". It is considered to be very common and is therefore a very well-studied bacteriophage. Its completely sequenced genome, precisely characterized protein structure, and described gene functions predispose T4 to be a model for phage biotechnology projects.

The world of biotechnology offers more and more advanced devices for molecular manipulation. This implies that in the future we will be able to change the properties of many organisms. The studies of bacteriophage T4 carried out so far have shown that such manipulations, including site-specific mutagenesis, may not only explain the functions of pivotal T4 proteins, but also significantly change the properties of the virus. It was shown that even slight modification of the genome may result in substantial changes of the mu-

tant's biology, which is why such studies should be continued. The phage-display technique employs the bacteriophage surface as a platform for displaying a variety of proteins, offering new solutions for various biological and chemical problems. It may, for example, be an effective and easy way of creating a site-directed drug delivery platform including the recently described system for tumor tissue targeting systems (Nilsson *et al*. 2000). The phage-display method was successfully applied on bacteriophage T4, which presents some advantageous features for this technique. The T4 capsid contains two inessential capsid proteins, Hoc and Soc, whose modification does not affect phage productivity, viability, or infectivity. The large capsid of T4 phage is able to bear relatively large proteins (also highly conformation-dependent proteins) without attenuation or propagation problems. This bacteriophage was also shown to be easily deprived of DNA, which substantially elevates its safety and simplifies its practical use, especially in *in vivo* applications. T4 was successfully used to display antigens of some important pathogens, including anthrax and HIV, constituting new strategies for vaccine development. All the studies showed that proteins on the T4 platform elicited immune response. Engaging inessential phage proteins in the T4 phage-display system enables separating *hoc* and *soc* fusion from the phage genome and allows *in vitro* capsid folding. These give the possibility of controlling the copy numbers of the presented protein(s). Furthermore, more than one antigen can be displayed at the same time in different combinations. Although we have focused on Hoc and Soc proteins, it is worth mentioning that another peptide was also used as a surface displaying platform. Modified T4 particles displaying 45 residues of the pre-S2 region of the hepatitis B virus in a 53-residue polypeptide chain were obtained. The display system was based on the minor T4 fibrous protein fibritin encoded by whisker's antigen control gene (*wac*) (Efimov *et al*. 1995). The studies showed that the use of T4 phage display is very flexible and easy. Although many studies have to be done, the initial results allow hope for beneficial effects of this technique and its broad application in the future.

The constantly developing bacteriophage therapy is becoming a very significant alternative to antibiotic treatment even in cases of antibiotic-resistant bacterial species (Gorski *et al*. 2007; Häusler 2006; Lorch 1999; Merril *et al.* 2003; Pirisi 2000; Stone 2000; Sulakvelidze *et al.* 2001; Weber-Dabrowska *et al.* 2000). The need for alternative strategies for antibacterial treatment generates a serious need for the means to control phage activity and for a better understanding of its mechanisms. The specificity of bacteriophages is still a challenge to investigators: its prediction and modifications may be the key to successful treatment. T4 has been shown to be easily manipulated, but some potent tools for specificity modifications (phage display) are still to be tested with this phage. Although we are not yet able to change this specificity freely, the studies which have been done so far showed that these modifications are really possible. Studies on interactions between T4 and the mammalian system showed that manipulating the T4 genome may result in important changes in the phage's properties. The time of phage circulation and its immunogenic properties may determine its utility in phage therapy. We hope that further progress in this field will allow obtaining sufficient data to apply phages as immunomodulators of immune response. These properties can be improved and adapted in further studies for medical and biological application of this bacteriophage.

We have shown that modified bacteriophage proteins may be applied in many medical issues. However, we also have to remember that we still do not know the ultimate properties and features of some native peptides. At the same time we know that many of them more frequently show closer sequence similarity with eukaryotic than with prokaryotic homologues (Bernstein and Bernstein 1989). Recently, the presence of immunoglobulin-like domains in Hoc peptide has been described. The similarity of sequences of Hoc gene and the eukaryotic IgSF may be significant considering the fundamental role of these molecules in the vertebrate immune system (Bateman *et al.* 1997). Further research may reveal the possibility of applying unmodified proteins or point out the direction of changes and show in what way they may be applied.

So far it seems that only small steps have been taken to exploit the molecular devices for adapting bacteriophages for clinical use. It is evident that this field of research will be greatly expanded in the nearest future, thus allowing for a better understanding of phages' immunobiology and its practical applications in biomedicine.

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