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## Phage engineering and the evolutionary arms race

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### Abstract

Phages are versatile agents for delivering a variety of cargo, including nanomaterials, nucleic acids, and small molecules. A potentially important application is treatment of antibiotic-resistant infections. All of these applications require molecular engineering of the phages, including chemical modification and genetic engineering. Phages are remarkably amenable to such engineering. We review some examples, including for controlled phage therapy. We suggest that the ability of phages to support extensive engineering may have evolutionary origins in the billions-year-old 'arms race' between bacteria and phages, which selects for sequences and structures that are robust in the face of rapid evolutionary change. This leads to high tolerance of both naturally evolved mutations and synthetic molecular engineering.

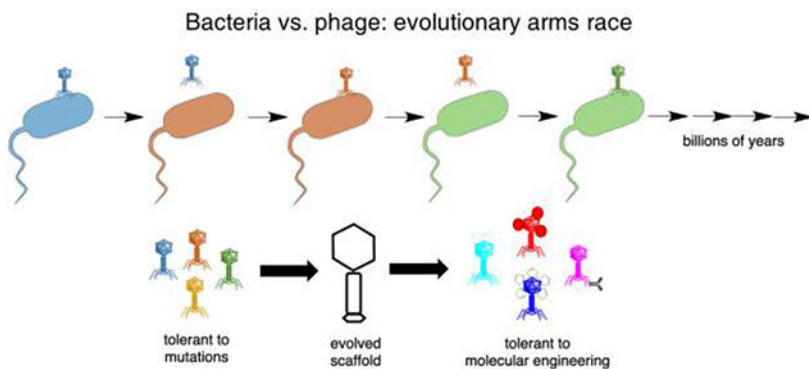
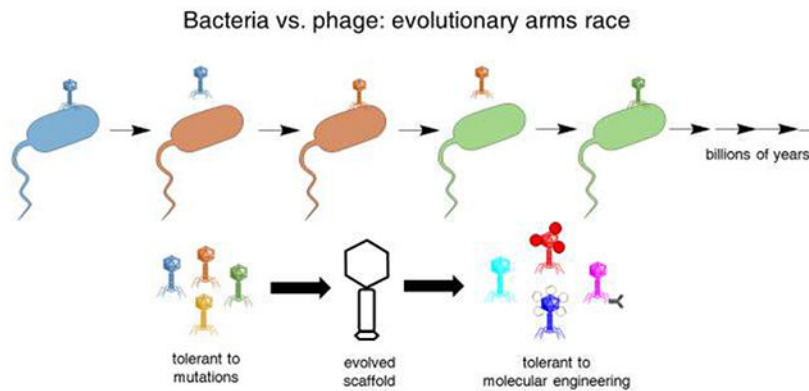
### Graphical abstract

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Nothing declared.



**Keywords**

Phage; phage therapy; arms race; red queen; robustness

**Introduction**

Phages are naturally evolved nanocarriers for delivering DNA and RNA. Virion production, survival, and attachment to host cells are all under selection to ensure phage propagation. These same features, along with ease of production in bacterial cell culture, make phages highly attractive as delivery vehicles for biomedical applications [1, 2]. As phage therapy returns to the attention of researchers concerned by the growing crisis of antibiotic-resistant infections, so do the associated biosafety concerns. Although phages are incapable of infecting eukaryotic cells, they can alter the properties of a bacterial infection and have public health implications through effects on their bacterial hosts. While, in an ideal situation, phages would lyse their bacterial hosts without other effects, many phages that infect pathogens have problematic behaviors from a clinical perspective. Phages that adopt a temperate rather than strictly lytic life cycle integrate into the host chromosome, enabling them to mediate gene transfer among bacterial cells through transduction. For example, phages of *S. aureus* transfer antibiotic resistance genes among their hosts [3]. Phages sometimes carry virulence factors, like phage CTX $\phi$ , which encodes and transmits a toxin gene that renders *V. cholerae* pathogenic to humans [4]. In addition to genetic effects, phage

particles can contribute physically to pathological infections, such as phage Pf4, whose virions create liquid crystalline biofilm compartments surrounding *P. aeruginosa* cells, increasing tolerance to antibiotics [5]. Moreover, Pf4 phage can trigger a maladaptive viral immune response, which suppresses clearance of the bacterial infection [6]. Because of the wide diversity of phages and the fact that the majority of phage sequences are uncharacterized, these negative effects are difficult to anticipate. Such concerns are intrinsic to a biocontrol strategy, namely that the use of a complex, evolving organism to eradicate another creates unpredictability in outcomes. While these risks may be acceptable in extreme circumstances [7], such unpredictability is undesirable in most therapeutic situations [8].

Several strategies can be used to avoid the potential problems, including engineering well-studied phages to have the desired tropism, adapting isolated phage proteins with lytic properties rather than whole phages, and engineering phages that 'self-destruct' through controllable nanomaterials [9]. These strategies raise the interesting issue of how amenable and robust phages are to engineering efforts. In the first section of this article, we review some illustrative examples of ways in which model phages are engineered to be delivery vehicles. These include a controlled version of phage therapy, in which phages would be enhanced by genetic or chemical controls to reduce potential side effects. Then, we reflect on why phages appear to be so tolerant to molecular engineering, a crucial element of any phage-based therapy.

## Engineering phages as delivery vehicles

Phages attach to bacterial host cells through receptor-binding proteins (RBPs) present on the virion particles, which have high affinity for a bacterial surface receptor and contribute to host specificity. If the therapeutic target is a bacterial species, RBPs themselves can be used for targeting. However, phages are also considered as delivery vehicles more broadly (e.g., to cancer cells), as the RBPs can be replaced or genetically engineered to bind the desired receptor. For example, a heterologous targeting peptide can be inserted as a fusion to a capsid protein. The targeting peptides might be derived from biological interactions, such as antibodies, or evolved from a library of random peptides by *in vitro* selection against the desired receptor (phage display). Cargo, such as nucleic acids, nanomaterials, therapeutic drugs, and diagnostic probes, can be attached through physical interaction or chemical conjugation with capsid proteins. Each virion contains hundreds to thousands of copies of capsid proteins, creating a large carrying potential. Solvent-accessible reactive groups, such as amino and carboxyl groups, can be utilized for functionalization [10, 11]. Some examples of phages engineered for cargo delivery are given in Table 1, illustrating a breadth of scaffolds, cargo, loading chemistry, and targeting applications.

Molecular engineering of phages tends to focus on a few model phages that infect *E. coli*, likely due to a paucity of established cloning techniques for other bacterial organisms and their phages. One of the most extensively investigated nanocarriers for controlled delivery and release is filamentous phages of *Inoviridae*, rod-shaped viruses including f1, M13 and fd (which are nearly identical to each other) [12, 13]. They are amenable to chemical modification, as the amino groups from lysine residues, the carboxylic acid groups of acidic

residues, and the aromatic groups of tyrosine residues of the major coat protein (pVIII) are potentially accessible and reactive. For example, carboxylic acid residues near the N-terminus (Glu2, Asp4, and Asp5) may be modified through EDC chemistry [14, 15]. Alternatively, primary amines of pVIII can be modified (e.g., by *N*-succinimidyl-*S*-acetylthiopropionate for thiolation); this approach has fewer reactive sites but might give more evenly distributed modification across the virion surface [9]. While chemical modification is simple, efficient and straightforward, it should be kept in mind that such treatments might also cause crosslinking among virions, denature proteins or alter cargo properties. Whether this occurs in practice must be seen on a case-by-case basis, but phages themselves appear to be remarkably tolerant to chemical modification.

Filamentous phages are also readily genetically engineered using well-established cloning methods [16]. By exchanging the receptor-binding domain of M13 (pIII) for that from a phage that naturally targets a different bacterial host, M13 can be engineered as chimeras to attach to a variety of different hosts, including the pathogens *P. aeruginosa* and *V. cholerae* [14, 15]. When coupled to gold nanorods (AuNRs), the chimeric phages can differentially kill specific strains of bacteria, as excitation of the AuNRs by near-infrared light induces surface plasmon resonance, whose energy is released locally as heat [17]. This effect (photothermal ablation) kills both cells and phages, equipping a well-studied phage with a 'self-destruct' strategy and the appropriate tropism to achieve controlled therapy [9]. Thus, use of a model *E. coli* phage need not limit the target range in certain applications. In addition, while the self-amplifying nature of phages could appear, at first glance, to be an attractive feature for phage therapy, self-replication may actually be undesirable due to the potential for unpredictable dynamics and interactions with the bacterial (or human) host [9]. A model *E. coli* phage may therefore have the further advantage of preventing replication on non-*E. coli* hosts. While phages are sometimes said to be very specific, in reality they vary in degree of specificity [18], depending on the level of conservation of the bacterial receptors and other interacting components. For example, phages capable of infecting multiple classes within the phylum Proteobacteria appear to be relatively common among environmental isolates [19], raising the possibility that non-model phages may have unexpected hosts. Such considerations compel development of strategies for controlled phage therapy based on well-studied model phages.

Genetic engineering, including phage display technology, can also be used to deliver cargo such as peptides, vaccines, and small molecules [20, 21]. For example, M13 can display collagen mimetic peptide on pIII along with a streptavidin-binding peptide on pVIII [22], to deliver streptavidin-linked fluorescent agents to the abnormal collagens expressed on human lung adenocarcinoma cells. Furthermore, filamentous phages are highly immunogenic, possibly due to the repeated structure of pVIII proteins and the relatively large dimensions. Therefore, whole phages can be used to enhance the immune response for vaccine delivery, and the vaccine itself may be stabilized against environmental damage by the phage-like particles [23]. Antigens may be expressed through genetic engineering [22] or by bioconjugation to the surface of phage particles [24]. At the same time, recent findings illustrating mechanisms by which another filamentous phage, Pf4, enhances the virulence of its host, *P. aeruginosa* [5,6], emphasize the importance of implementing multiple levels of

control over the phage, such as self-destruction by nanomaterials [15] and cross-species barriers that prevent completion of a replication cycle on the targeted pathogenic species.

Another prominent model phage is MS2, which has very small virions (~27 nm in diameter) with a symmetric icosahedral capsid containing 180 copies of the coat protein [25]. Unlike the ssDNA-containing filamentous phages, MS2 phages contain a single-stranded RNA genome (family *Leviviridae*), making genetic engineering less straightforward [26]. However, MS2 capsids have 32 pores that allow access to the inside of the capsid [27], enabling chemical modification of the interior as well as exterior surfaces. For example, using orthogonal bioconjugation chemistry, the exterior can be modified with a targeting reagent while the cargo is attached to the interior. In one example, fluorescent dyes were attached to the interior of genome-free MS2 capsids by site-specific alkylation, while the outer surface was decorated with DNA aptamers by a NaIO<sub>4</sub>-mediated oxidative coupling reaction [28]. The resulting particles could bind to tyrosine kinase receptors on a cancer cell line and were readily endocytosed. For a cytotoxic application, the MS2 interior was modified with porphyrins that generate singlet oxygen upon illumination, such that the capsids could deliver photodynamic therapy to targeted cancer cells [29]. Peptides are commonly used for targeting, such as peptide SP94, which enables selective delivery of a variety of cargo to human hepatocellular carcinoma cells [30]. Since the natural cargo of MS2 phage is single-stranded RNA, it is also a promising nanocarrier for RNA delivery [31, 32]. In one case, engineered MS2 particles activated osteoblast differentiation by delivering mRNA for transcription factors into bone-marrow mesenchymal stem cells [33], illustrating how MS2 can be considered for delivery of a transient, though limited, load for gene therapy.

When delivery of large amounts of genetic cargo is needed, one must turn to larger phages [34]. Among these is lambda phage, whose temperate lifestyle became a classic subject of study during the early days of molecular biology. Lambda phages contain a double-stranded DNA genome of 48,502 bp and have the 'lunar lander' shape characteristic of the large taxon *Caudovirales*, including an icosahedral head, tail, and tail nanofibers [35]. An interesting potential application is DNA vaccine delivery [36, 37], although whether phages possess sufficient advantages over eukaryotic viruses for gene delivery to mammalian cells is unclear. Nevertheless, gene delivery to prokaryotic cells could be a natural niche. For applications that require peptide display rather than gene transfer, lambda phages are particularly useful for large peptides [38, 39], where expression can be two to three orders of magnitude more efficient than M13-based vectors [40, 41]. This difference stems from the abundance of the coat proteins capable of accommodating large peptides; protein gpD on lambda phage is present in ~420 copies per virion, while g3p on M13 phage is present at ~5 copies per virion (with g8p on M13 being essentially unable to display peptides longer than 16 aa) [42]. For example, lambda phage displaying a HER2/neu peptide on the capsid activated cytotoxic T-lymphocytes against a murine breast cancer model [43]. Thus, lambda phage may be useful as delivery agents for larger cargo including both genes and antigens. Other *Caudovirales* phages, including T4, T7, and P22, are not reviewed here, but have also been used for display and engineering.

## Evolutionary robustness and molecular engineering

Given the examples above, phages appear to be remarkably easy to modify, both by genetic modification such as creation of fusions to capsid proteins, or by chemical modification such as bioconjugation to small molecules or nanoparticles. In our own work [9, 14, 15], we have been struck by the fact that every chimeric capsid protein we created resulted in a functional construct, despite the fact that the proteins had no detectable homology to each other. The fact that virions retain the ability to assemble and attach after quite substantial perturbations, including decoration by both macromolecules and small molecules, points toward high robustness of these activities during engineering. This robustness is quite different from, for example, the challenges encountered when engineering antibodies, another class of biomolecules that is under analogously strong selection for attachment [50, 51].

What might drive such robustness to engineering? Robustness itself is a trait under selection. In RNA viruses, which exhibit very high mutation rates (nearly one mutation per generation), strains selected for environmental robustness, such as tolerance of high temperatures, also exhibit increased tolerance of mutations, i.e., increased evolvability [52, 53]. At a molecular level, protein folds that are more environmentally stable appear to be also more stable in the face of genetic changes. Phages are under direct selection for environmental stability, as they must survive the extracellular environment and its thermal, chemical, and physical fluctuations. On top of this, phage proteins are under further selection to tolerate mutations. As bacteria evolve resistance to phages, and phages evolve to counter resistance, the 'evolutionary arms race' between phage and bacteria drives positive selection resulting in rapid evolution and divergence (Figure 1). However, the proteins must fold into similar structures to maintain the virion, despite the rapid pace of evolutionary change. Thus, the protein scaffolds must have evolved to be tolerant to mutations. Yet genetic mutations are only one type of chemical modification. These selective pressures appear to have also led to tolerance of chemical modifications more generally, such as those used for loading cargo, including fairly imprecise, widespread modifications, and to fusion proteins and conjugation to large structures such as nanoparticles. Selection for general adaptability to novel conditions has also been observed in viral adaptation to rapid environmental changes [54]. Thus, natural selection appears to have driven phages to exhibit traits that are particularly favorable for molecular engineering.

## Conclusion

While phages have important advantages as delivery vehicles, especially when targeting bacterial cells (e.g., controlled phage therapy), important challenges remain. Circulation times may be short as the phages are cleared by the reticuloendothelial system, although PEGylation of the phage particles and genetic engineering can extend these times [55, 56]. Certain phages may have negative consequences for the human host, such as by inappropriate stimulation of an antiviral response [6] or through release of endotoxins [57], or may lead to undesired gene exchange among bacteria, as discussed above. Controlled phage therapy is needed to prevent such consequences, either by directly managing dosing [9], or engineering a lysis-deficient phage [58-60] or a lysogenic phage that expresses lethal but non-lytic proteins [61]. There is reason for optimism that these challenges can be met by

molecular engineering, given the extraordinarily robust and tolerant architecture of phages resulting from billions of years of an evolutionary arms race. While phage scaffolds may be tolerant to engineering, genetic engineering can be technically quite challenging with phages and is itself a rapidly growing area of research [62, 63]. An increasing body of work demonstrates the versatility of phages for carrying diverse cargos while targeting can be engineered essentially orthogonally. Continuing successes in engineering phages paves the way for realizing the long-held dream of safely utilizing the exquisitely honed mechanisms phages possess to 'hunt' their bacterial hosts.

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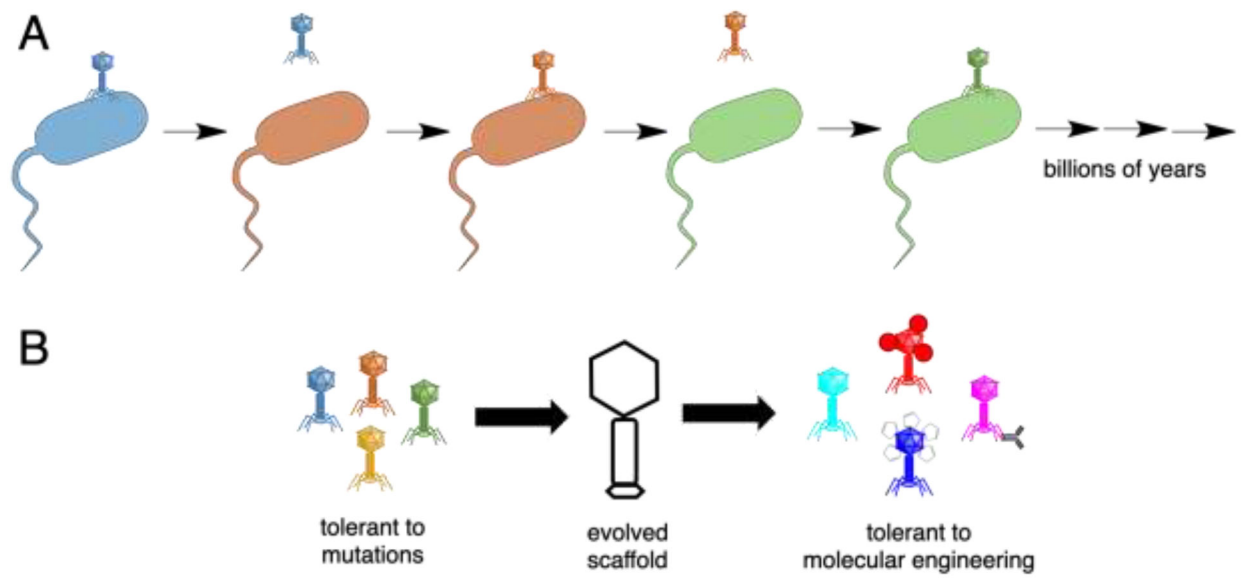
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### Highlights

- Phages can target delivery of cargo, including nanomaterials, drugs, and genes
- Molecular engineering is essential to applications such as controlled phage therapy
- The phage-bacteria arms race causes rapid evolution and tolerance of mutations
- Phages are therefore remarkably tolerant of chemical modification in general



**Figure 1.**

The evolutionary arms race between bacteria and phage drives a rapid evolutionary pace (A). The resulting phage scaffolds were presumably selected for tolerance to mutations. A correlated trait is tolerance to other chemical modifications, such as those used in molecular engineering (B).

Table 1.

Examples of phages engineered for cargo delivery.

Phage/Component	Cargo	Loading technique	Targeting	Application	Reference
<b>M13</b>	Gold nanorods	Thiolation of g8p by chemical modification to form Au-S bonds	Bacterial receptor-binding proteins displayed on g3p	Photothermal lysis of <i>P. aeruginosa</i> biofilms, controlled phage therapy	[9]
<b>fd-tet phage displaying 9-mer peptide</b>	Small interfering RNA (siRNA)	Interaction of positively charged C-terminal end and negatively charged phosphate of siRNA	Tumor-specific peptides isolated from phage library	Delivery to breast cancer cells, leading to specific silencing of the model gene GAPDH	[44]
<b>T7</b>	Fluorescent protein	Displayed as fusion to capsid protein by phage display	Gold-binding-peptide displayed on the tail protein; gold nanoparticles decorated with specific oligonucleotide	Counting miRNA by the naked eye in a Petri dish	[45]
<b>M13</b>	Exogenous proteins	Biotin conjugation to capsid; streptavidin fusion to cargo	Prostate cancer cell-penetrating peptide displayed on g3p	Delivery of functional imaging protein and enzymes	[46]
<b>P22 capsid</b>	Gd <sup>III</sup> -chelating agents	Thiol-maleimide Michael-type addition	Passive targeting	Visualizing murine intravascular system by <i>in vivo</i> magnetic resonance imaging	[47]
<b>Lambda</b>	Mammalian expression cassette encoding firefly luciferase	Genetic engineering	An integrin-binding peptide displayed on phage	<i>In vivo</i> lambda phage-mediated gene delivery and expression	[48]
<b>MS2 capsid</b>	Capsids radiolabeled with <sup>64</sup> Cu	Antibody conjugated to exterior by nitrophenol-amine reaction; cargo loaded by metal chelator maleimide reaction with internal cysteines	Anti-EGFR antibody targeting breast cancer cells	<i>In vivo</i> PET/CT imaging of tumor xenografts in mice	[49]