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SpyPhage: A Cell-Free TXTL Platform for Rapid Engineering of Targeted Phage Therapies

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Abstract

The last decade has seen the emergence of multi-drug resistant pathogens as a leading cause of deaths worldwide, reigniting interest in the field of phage therapy. Modern advances in the genetic engineering of bacteriophages have enabled several useful results including host range alterations, constitutive lytic growth, and control over phage replication. However, the slow licensing process of genetically modified organisms clearly inhibits the rapid therapeutic application of novel engineered variants necessary to fight mutant pathogens that emerge throughout the course of a pandemic. As a solution to this problem, we propose the SpyPhage system where a "scaffold" bacteriophage is engineered to incorporate a SpyTag moiety on its capsid head to enable rapid post-synthetic modification of their surfaces with SpyCatcher fused therapeutic proteins. As a proof of concept, through CRISPR/Cas-facilitated phage engineering and whole genome assembly, we targeted a SpyTag capsid fusion to K1F, a phage targeting the pathogenic strain *Escherichia coli* K1. We demonstrate for the first time the cell-free assembly and decoration of the phage surface with two alternative fusion proteins, SpyCatcher-mCherry-EGF and SpyCatcher-mCherry-Rck, both of which facilitate the endocytotic uptake of the phages by a urinary bladder epithelial cell line. Overall, our work presents a cell-free phage production pipeline for the generation of multiple phenotypically distinct phage with a single underlying "scaffold" genotype. These phages could become the basis of next-generation phage therapies where the knowledge-based engineering of numerous phage variants would be quickly achievable without the use of live bacteria or the need to repeatedly license novel genetic alterations.

1 Introduction

A major triumph for humanity has been the discovery and widespread use of antibiotics to fight bacterial infections. Indeed since their first applications at the beginning of the last century, antibiotics have revolutionised modern medicine and been effective in saving millions of lives.[1] However, as we close in on the third decade of the 21st century, the irresponsible administration of antibiotics has led to the now prevalent problem of antimicrobial resistance (AMR).[2] It is predicted deaths as a result of AMR will reach 10 million by the year 2050, threatening to usher in a post-antibiotic era.[3] While a multifaceted approach to the present problem is required, a key strategy in the fight against this silent pandemic is the development of new antimicrobial therapies. To this end, in recent years there has been a resurgence in the field of phage therapy, with research efforts intensifying on the engineering, production and testing of the antimicrobial efficacy of these natural bacterial predators.[4]

Bacteriophages (phages) are viruses that infect bacteria and are the most abundant organism on earth.[5] They possess a relatively narrow host range and in the case of lytic phages, have the ability to cause rapid lysis and subsequently kill their host.[1] It is this natural predator: prey phenomenon that modern phage therapy attempts to harness in the treatment of antimicrobial resistant bacteria. While numerous examples of successful clinical use of phage for the treatment of AMR infections have now been demonstrated, there remain significant technical challenges that must be overcome to enable their widespread adoption.[6–8] The unique pharmacological properties of phage, the potential for bacterial targets to develop resistance to phage cocktails and the ability of the mammalian innate immune system to recognise and clear viral particles present major challenges in the administration of clinically effective phage doses.[6] Nonetheless, modern advances in synthetic biology such as cell-free transcription/translation (TXTL), CRISPR gene editing and whole genome assembly, hold great promise in enabling researchers to overcome the current bottlenecks in phage therapy research.[9]

It has long been envisioned that the ability for researchers to generate whole phage genomes from synthetic oligonucleotides could allow for unprecedented reductions in time and cost of the engineering of new phage therapies.[10–12] In addition, the ability to boot-up bacteriophages from their genomes within cell-free TXTL reactions would allow for the production of high titre phage stocks with low levels of contaminants.[9] Further still, cell-free TXTL systems produced from non-pathogenic chassis organisms could be engineered to enable the *in vitro* assembly of phage which naturally require pathogenic hosts for their propagation.[13] Nonetheless, in the ten years since Noireoux and co-workers defined the modern gold standard protocols for TXTL-based phage assembly, research thus far has primarily focussed on defining the *in vitro* assembly conditions for different phages and the use of phage assembly as a benchmark of the power of a particular *in vitro* TXTL system.[14–16]

In the present work, we demonstrate the utility of cell-free TXTL for phage therapy by outlining a generally applicable phage engineering pipeline for the creation of next-generation phage-based therapies. As a proof of concept, we focus on the rapid engineering of phage K1F for the treatment of infection by the gram-negative pathogen *E. coli* K1 which is responsible for a wide range of diseases including sepsis, urinary tract infections, inflammatory bowel disease and neonatal meningitis.[17–19] The latter is a particularly devastating disease with a 10% mortality rate in affected infants, while 20-50% of neonatal meningitis survivors develop complications such as seizures, cognitive deficiencies, and motor abnormalities.[17]

As with any phage therapy, the administration of K1F phage to treat the aforementioned conditions would be followed by the rapid clearance of the administered phage by the innate immune mononuclear phagocyte system (MPS) of the patient.[9] In addition, it has been hypothesised that the adaptive memory antibody response may be implicit in preventing the re-administration of a particular phage therapy cocktail within the same patient.[20] As

such, strategies to evade the MPS and allow for long-circulating phage therapies are needed to enable enhanced therapeutic efficacy. To facilitate a simple solution to these problems, we demonstrate the first known incorporation of the SpyTag protein on to the capsid head of the K1F phage *via* homologous recombination and CRISPR/Cas9 mediated selection, as well as through whole genome assembly. The SpyTag protein is one-half of the powerful protein conjugation pair termed the SpyTag/SpyCatcher system.[21] The system is derived by splitting the *Streptococcus pyogenes* fibronectin-binding protein FbaB into two functional domains, *viz.* SpyTag and SpyCatcher, that form a spontaneous isopeptide bond between Lys and

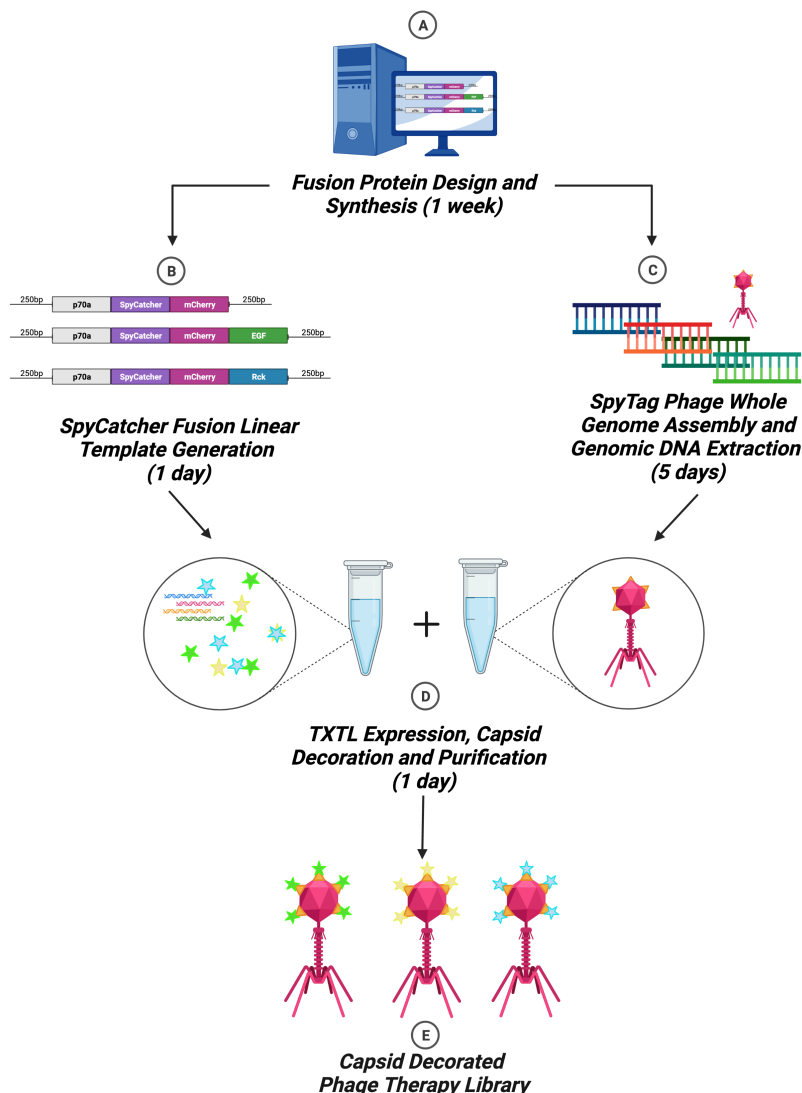

Figure 1: SpyPhage Engineering Pipeline. A lead time of 1 week is allocated for the design and synthesis of linear TXTL templates for therapeutic SpyCatcher decoration proteins as well as a gene fragment coding for SpyTag fusion to the minor capsid protein of K1F phage (A). Linear TXTL templates for capsid decoration proteins are PCR amplified from synthesised gene fragment templates (B). Rapid phage engineering is enabled by an 8-piece whole genome assembly of SpyTagged K1F using the ordered gene fragment and a further 7 fragments generated from the wild-type K1F genome *via* PCR (C). SpyTagged K1F phage and capsid decoration proteins expressed from cell-free TXTL reactions are combined to enable post-translational capsid decoration *via* SpyTag-SpyCatcher (D). In this manner, the engineering pipeline generates multiple phenotypically distinct phage with the same underlying scaffold genotype (E).

Asp residues.[22] This irreversible covalent interaction is an ideal method to attach therapeutic proteins to the capsid head of SpyTagged K1F phage and provides a method for simple and efficient capsid decoration to create phenotypically distinct phage for clinical use without further genetic engineering.

As validation of the utility of the phage engineering pipeline proposed, we demonstrate the assembly and capsid decoration of SpyTag bearing K1F phage in an *E. coli* cell-free TXTL system at clinically relevant titres of 10^9 PFU/mL. We focussed on creating variants of SpyTagged K1F with post-translational capsid decorations that could alter the mechanism of phage entry into human cells and allow for prolonged bioavailability *in vivo*. To this end, we decorated the capsid head of cell-free TXTL assembled SpyTagged K1F with epidermal growth factor (EGF) or the *Salmonella* outer membrane protein Rck (resistance to complement killing), each of which facilitated the entry of phage *via* endocytosis through the EGF receptor (EGFR). Starting from synthesised DNA fragments for therapeutic proteins and phage genome fragments, the engineering pipeline described herein enabled the cost-effective and time-saving production of three phenotypically distinct phage in 5 working days (Figure 1).

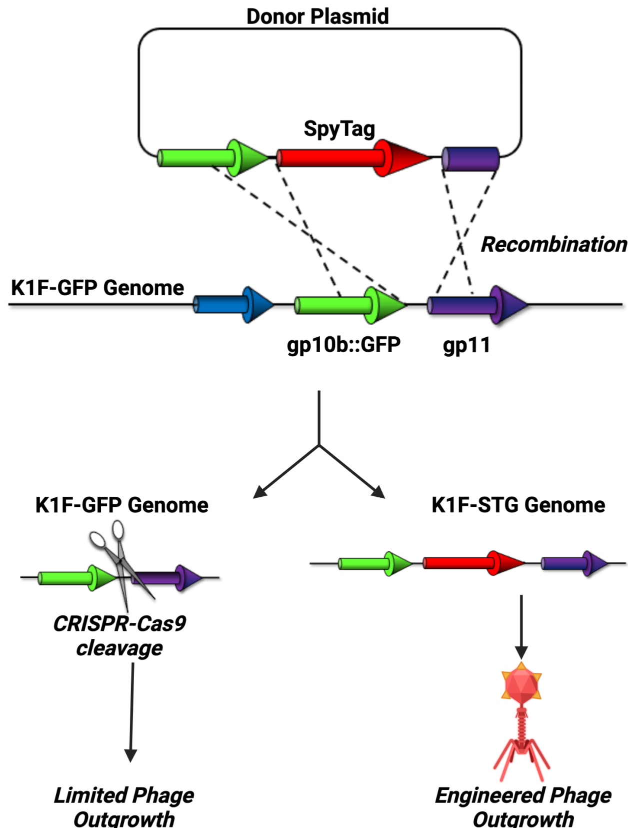

Figure 2: Phage engineering *via* homologous recombination. K1F-GFP is further engineered to incorporate a SpyTag fusion to GFP on gene10b *via* homologous recombination to create K1F-STG. CRISPR/Cas9 selection is used to isolate K1F-STG from a mixed population by cleaving the K1F-GFP genome.

2 Results and Discussion

Engineering SpyTagged K1F Phage

The goal of this work was to combine modern tools of genetic engineering with advances in cell-free TXTL to create a platform technology for the rapid engineering of bacteriophages for targeted phage therapies.

To illustrate the benefits of our novel method, we implemented the first known engineering of K1F phage to incorporate SpyTag gene fusions onto the capsid head *via* two distinct engineering methods. In the first instance, homologous recombination and CRISPR/Cas9 selection was used to insert the SpyTag gene onto the phage head to enable simple capsid decoration of K1F phage (Figure 2). We had previously demonstrated that the fusion of GFP to the minor capsid protein to create a fluorescent variant of K1F Phage (K1F-GFP) did not affect the fitness of the engineered phage.[19] Therefore, we chose to further engineer K1F-GFP to incorporate SpyTag fused to GFP to create K1F-GFP-SpyTag (K1F-STG). The new K1F-STG phage carries the gene10b::GFP-SpyTag fusion construct, whereby fusion of SpyTag to GFP is mediated by a Gly-Ser-Gly-Glu-Ser-Gly semi-flexible linker (Figure S2). These modifications facilitate downstream post-translational capsid modifications and simple visualisation under fluorescence microscopy. Sequencing data for K1F-STG clonal isolates are presented in Figure S11.

Although homologous recombination and

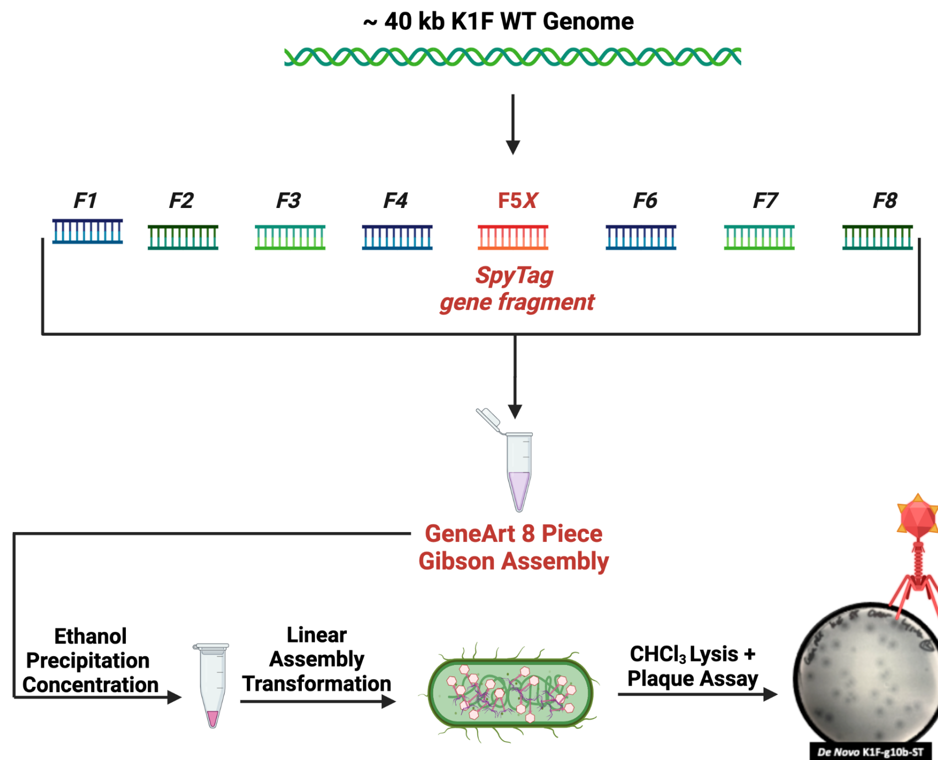

Figure 3: Whole genome assembly. K1F-STB was created *via* an 8-piece Gibson assembly of the entire phage genome. This versatile method for phage engineering results in the production of only SpyTagged K1F phage, negating the need for downstream selection.

CRISPR/Cas9 selection is a useful tool for the engineering of bacteriophages, each modification requires the creation of a modification-specific set of donor and CRISPR/Cas9 selection plasmids. In contrast, rationally designed whole genome assembly offers a greater versatility through which multiple modifications can be made simultaneously, thereby reducing the costs and the time required in phage engineering.[23]For this reason, we attempted the whole genome assembly of a K1F phage bearing SpyTag on the minor capsid protein *via* Gibson assembly, and transformation of the assembly mix into *E. coli* to produce engineered phage (Figure 3).

The design of the assembly ensures that gene10 of the K1F genome resides within the 5th fragment of an 8-fragment genome assembly (Figure 3). By limiting the size of the 5th fragment to less than 3000 base pairs in length, desired modifications can be made to gene10 and the entire fragment can be commercially synthesised as a gene fragment to be used directly in the assembly reaction. Following Gibson assembly reaction completion, and transformation of the assembly mix into a DH5a cell line, engineered phage can be booted up from the newly assembled genome *in vivo*. This enabled us to create K1F-gene10b::SpyTag (K1F-STB) via whole genome assembly within 2 working days (Figure S4). Sequencing data for K1F-STB clonal isolates generated from whole genome assembly are presented in Figure S12.

As fragment 5 was synthesised and all other fragments were gel extracted following a PCR amplification from the wild-type genome (Figure S3), our method resulted in no carry over of wild-type genome into the Gibson assembly reaction. Hence, all phages synthesised are only as a result of a successful whole genome assembly and all contain the desired genetic modification. Interestingly, we found that a successful phage genome assembly can be achieved with gel extracted fragments using the GeneArt Gibson Assembly EX system

but not using the NEBuilder HiFi Gibson Assembly Master Mix. We believe that this is a result of the higher sensitivity of the NEBuilder kit to inhibitory agents carried over from the gel extraction procedure and due to the overall higher efficiency of the two-step assembly process in the GeneArt System.[24] The design of the whole genome assembly to isolate the entirety of the gene to be modified on to a single commercially synthesised fragment enables a user-defined plug and play approach for the incorporation of any gene of interest into a phage genome using a rapid 2-day protocol.

While the above described method is indeed useful for the rapid engineering of recombinant phage with desired modifications, it still relies on an initial *in vivo* propagation step to obtain engineered phage. To bypass this step, attempts were made to boot up K1F-STB from the newly assembled genome within a TXTL reaction. In such instances, the concentrated and purified assembly mix was used as the template for a phage assembly TXTL reaction, though these attempts were unsuccessful (data not shown). It was thought that the efficiency of the Gibson assembly did not lead to sufficiently high levels of fully assembled phage genomes to enable TXTL-based boot up of engineered phage. The optimisations of such strategies would merit future work to facilitate a complete *in vitro* phage engineering pipeline. Nonetheless, as with K1F-STG engineered by homologous recombination, K1F-STB phage engineered by whole genome assembly were genetically stable as evidenced by the ability to repeatedly propagate the engineered phage without the loss of the recombinant sequence.

Cell-Free TXTL Assembly of K1F

We report the first known demonstration of the assembly of K1F phage variants in a TXTL system. A salient feature of this result is that the time reductions are achieved by preparing genomic templates for TXTL from PEG-purified phage instead of using CsCl-purified phage. As a benefit, it ensures that there is no carryover of CsCl, which can be toxic, into phage therapy.[25]

The expression of genome-sized DNA programs for phage production in TXTL is initiated by endogenous sigma factors present in the prepared extract.[26] In the case of K1F phage, the genome encodes a K1F-specific RNA polymerase (RNAP) under the control of both a sigma factor promoter and a downstream K1F RNAP promoter. Once expressed, the K1F RNAP is available to transcribe phage-specific genes located throughout the ~40 kb genome, as well as to regulate the transcription of the K1F RNAP gene itself.[27] The reliance on host sigma factors for the initiation of phage gene transcription requires the production of a powerful cell extract rich in sigma factors. To achieve this, we established an *E. coli* crude extract preparation method that combined three different lysis methods (autolysis, lysosome-assisted lysis and sonication) for the reproducible production of crude cell extract with a concentration of 35 mg/mL (Figure S5).[28–31]

As with any cell-free reaction (CFR), the

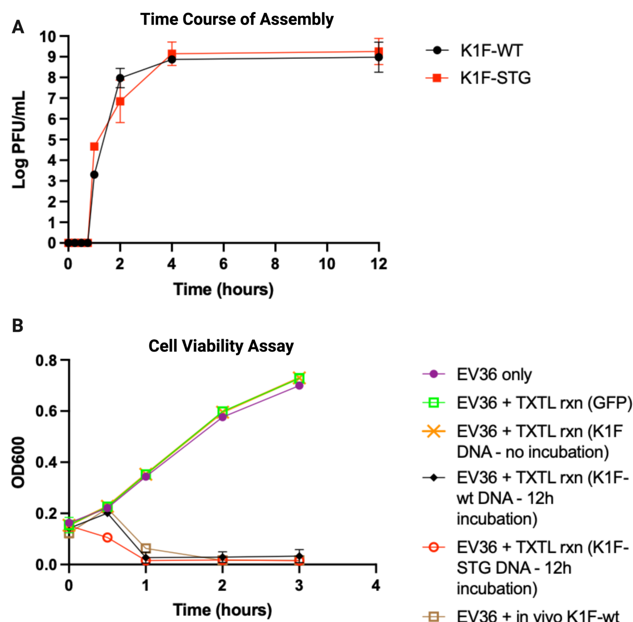

Figure 4: Cell-Free TXTL Assembly of K1F Variants. K1F-WT and K1F-STG were assembled in TXTL reactions with maximum phage titres of 10^9PFU/mL being reached within 5 hours for both phages (A). Phage assembled in cell-free reactions incubated for 12 hours were able to kill its bacterial host *E. coli* EV36 to the same effect as that propagated and purified from bacteria (*in vivo* K1F-WT) (B).

quality of the DNA template is a principal factor in the successful expression and assembly of bacteriophages *in vitro*.[32] Classical protocols require the generation of highly pure phage stocks using either CsCl or sucrose gradient purification, from which high concentration genome stocks are extracted.[30] This trend in using gradient purified phage for genomic DNA extraction is common due to its use for the preparation of highly pure samples for metagenomic sequencing.[25] However, the isolation of bacteriophages using such methods require complex gradient setup and long-duration high-speed ultracentrifugation.[25] To simplify this workflow, we propagated K1F phage to obtain phage lysates which were purified using a robust PEG precipitation protocol (Supporting Information S5). PEG-purified phage were subsequently treated with an equal volume of chloroform and genomic DNA was extracted using phenol:chloroform. Our method enabled the reproducible generation of high concentration K1F genomic stocks which were free from bacterial and phage contaminants (Table S8).

We set the additive concentrations of our CFRs for K1F phage assembly to match that of previously established parameters for T7 phage due to their structural similarities.[16]In this manner, we utilised our cell-free system (CFS) for the concurrent expression of 58 different proteins and their assembly into virulent K1F phage particles at clinically relevant titres of 10^9PFU/mL without further additive optimisations.[33] This titre was comparable to a maximum titre of 10^{11}PFU/mL achieved using the highly optimised commercially available myTXTL kit. We observed that both K1F wild type (K1F-WT) and K1F-STG followed a similar time course of assembly, with virulent particles detectable in the CFR after 1 hour and maximum viral titre being reached within 5 hours (Figure 4A). The ability of cell-free assembled K1F-WT and K1F-STG to kill their host was comparable to the same phage produced and purified from bacteria (Figure 4B).

Altering the Mechanisms of Phage Entry into Human Cells

Following the engineering of SpyTagged K1F variants and determination of their assembly conditions in our CFS, their utility as a scaffold phage for use in phage therapy was explored. The method described herein facilitates an easy to implement strategy for the TXTL-based capsid decoration of SpyTagged scaffold phage to produce multiple phenotypically distinct phage with the same underlying genotype. As a result, it may be hoped that regulatory approval would be easier to obtain for it. In this instance the SpyTagged K1F phage acts as a scaffold to which other proteins of interest fused to SpyCatcher can be post-translationally coupled, thereby allowing for the rapid screening of therapeutic proteins without the need to further engineer the phage. This is of particular importance due to the difficulty in engineering the capsid of phage to incorporate large proteins, whereas the engineering of a scaffold phage i.e. a phage incorporating a SpyTag sequence on its capsid head is comparatively easier due to the small size of the SpyTag protein.[34] As a demonstration of the utility of cell-free TXTL-based phage engineering as a platform technology for phage-based therapies, we chose to decorate the capsid head of cell-free TXTL assembled K1F-STG with TXTL expressed SpyCatcher fusion proteins that could alter the mechanism of phage entry into human cells.

We had previously demonstrated that the phage K1F-GFP invades human T24 epithelial cells *via* phagocytosis and are subsequently degraded *via* LC3-assisted phagocytosis.[19] However, in order to increase the success of phage therapy, it is advantageous to direct phage entry into human cells *via* the endocytic pathway such that phage that enter human cells are able to evade phagocytic degradation and thereby increase their bioavailability.[35–37] A potential mechanism to enable endocytic entry is through the capsid decoration of phage with epidermal growth factor (EGF), a 7 kDa cytosolic protein that plays a critical role in cell growth, signal transduction and differentiation.[35] EGF exerts its mitogenic effect via binding to its cognate receptor, the EGF receptor (EGFR), where the binding of the EGF ligand to EGFR is highly specific and induces a conformational change of the receptor at the cell membrane.[38] The resul-

tant binding induces receptor dimerization via trans-autophosphorylation of tyrosine residues of the opposed intracellular domains, and the receptor/ligand complex is subsequently internalized via clathrin-mediated endocytosis.[39]

In addition to EGF, the outer membrane protein Rck of *Salmonella*, can also bind to the EGF receptor.[40] Indeed, the Rck protein is implicit in the induction of *Salmonella* entry into human cells as it mimics natural host cell ligands and triggers engulfment of the bacterium via the zipper mechanism of entry through the EGF receptor.[41]Furthermore, it has been demonstrated that the Rck protein plays a role in the intracellular virulence of *Salmonella* through conferring serum resistance as a result of the binding of Rck to complement regulatory protein factor H (fH).[42–44] We therefore hypothesised that the post-translational capsid decoration of K1F-STG with EGF or Rck would allow entry into human cells *via* the EGF receptor, with Rck decoration providing the added benefit of serum resistance by preventing opsonisation of the administered phage.

Prior to testing the proposed EGF and Rck decorations, it was first necessary to investigate

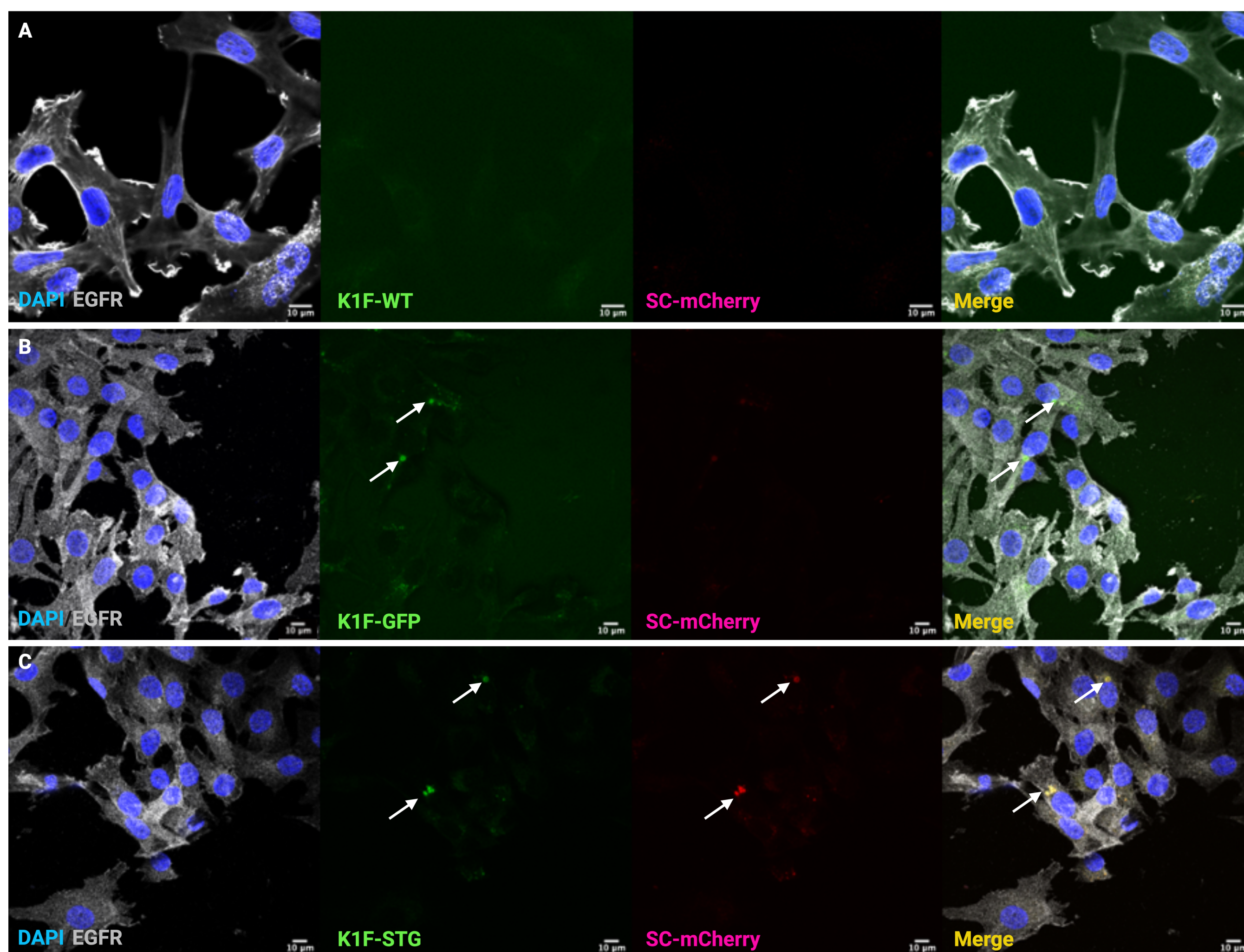

Figure 5: Capsid decoration proof of concept. Immunocytochemistry fluorescence microscopy of bacteria assembled K1F variants (green) decorated with SpyCatcher-mCherry (red) in T24 human urinary bladder epithelial cell line stained with DAPI (blue) and antiEGFR (grey). K1F-WT and K1F-GFP does not covalently bind with SpyCatcher-mCherry due to the lack of SpyTag on the capsid head (Panel A and B). K1F-STG covalently binds SpyCatcher-mCherry and carries capsid decoration into human cells (Panel C). SpyCatcher-mCherry decorated K1F-STG does not interact with EGF receptor.

any trophic effect of SpyCatcher-mCherry alone to the EGF receptor. This is an important consideration due to the fact the SpyTag/SpyCatcher system is itself derived from the second immonoglobulin-like collagen adhesion domain (CnaB2) of FbaB, a protein that facilitates the uptake of *S. pyogenes* by endothelial cells.[45] For these experiments, all variants of K1F (WT/GFP/STG) were propagated and PEG purified from *E. coli* EV36 bacterial lysates and capsid decoration was attempted using purified SpyCatcher-mCherry from *E. coli*. Following their creation, decorated phage were used to infect T24 human urinary bladder epithelial cells stained with a primary antibody for the EGF receptor. As expected, SpyCatcher-mCherry did not couple to K1F-WT or K1F-GFP as they lack the SpyTag protein fusion on their capsid head (Figure 5A, 5B). In both instances, occasional diffuse signals (background fluorescence) for the mCherry channel was observed, thus demonstrating the specificity of the interaction of SpyCatcher to its fusion partner SpyTag. In contrast, clear evidence of post-translational coupling of SpyCatcher-mCherry to the capsid head of K1F-STG and subsequent translocation of coupled phage into T24 human urinary bladder epithelial cells was observed (Figure 5C). In such instances, fluorescence microscopy revealed punctate and co-localised signals of GFP and mCherry from GFP-SpyTag fused to gene10b on the K1F-STG capsid and SpyCatcher-mCherry post-translationally coupled to GFP-SpyTag on gene10b, respectively. Notably, the decoration of K1F-STG with SpyCatcher-mCherry alone did not confer tropism of the decorated phage to the EGF receptor (Table 1).

Next, we sought to alter the mechanism of entry of K1F-STG by decorating its capsid with EGF or Rck. To this end, linear DNA templates were designed to encode SpyCatcher-mCherry fusions to EGF or Rck proteins under the control of the p70a promoter (Figure S6). These linear DNA templates were PCR amplified from

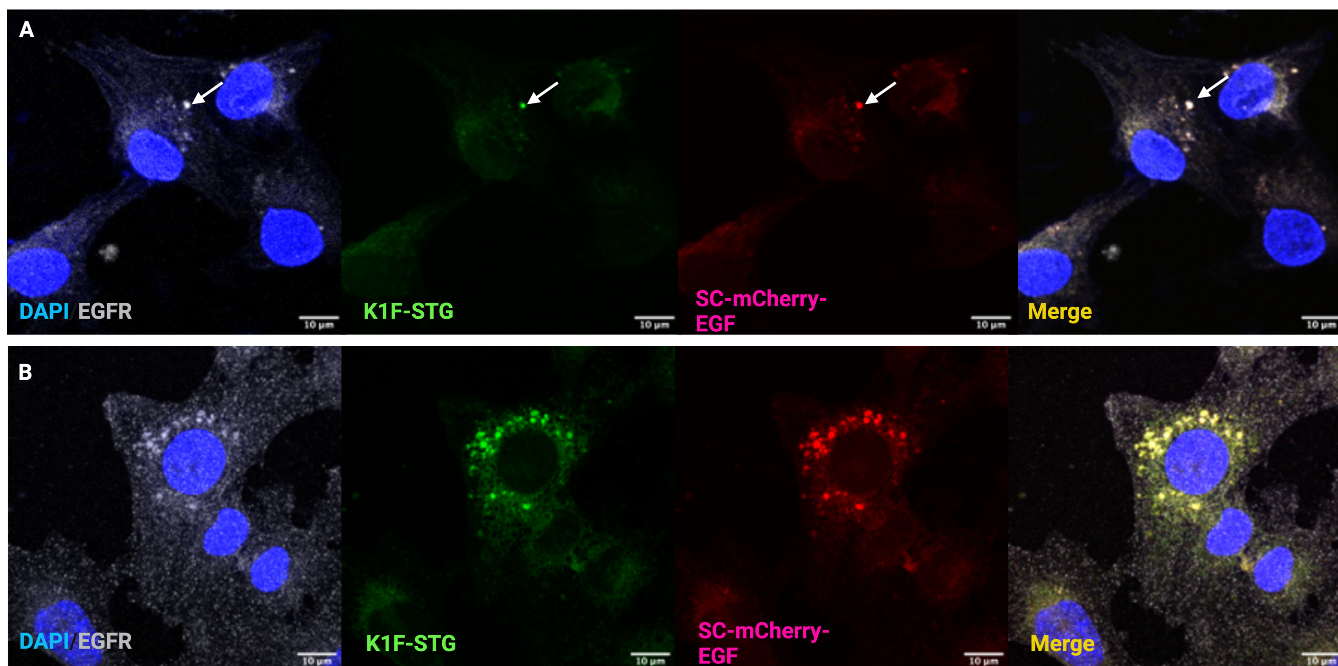

Figure 6: Altering mechanism of phage entry with SpyCatcher-mCherry-EGF capsid decoration. Immunocytochemistry fluorescence microscopy of cell-free TXTL assembled K1F-STG (green) decorated with SpyCatcher-mCherry-EGF (red), to enable activation of the EGF receptor (grey) in a T24 human bladder epithelial cell line stained with DAPI (blue). White arrows indicate the interaction of EGF decorated phage with EGFR (A). Activation of EGFR leads to endocytic entry as demonstrated by the presence of endosomes in the cytosol (B).

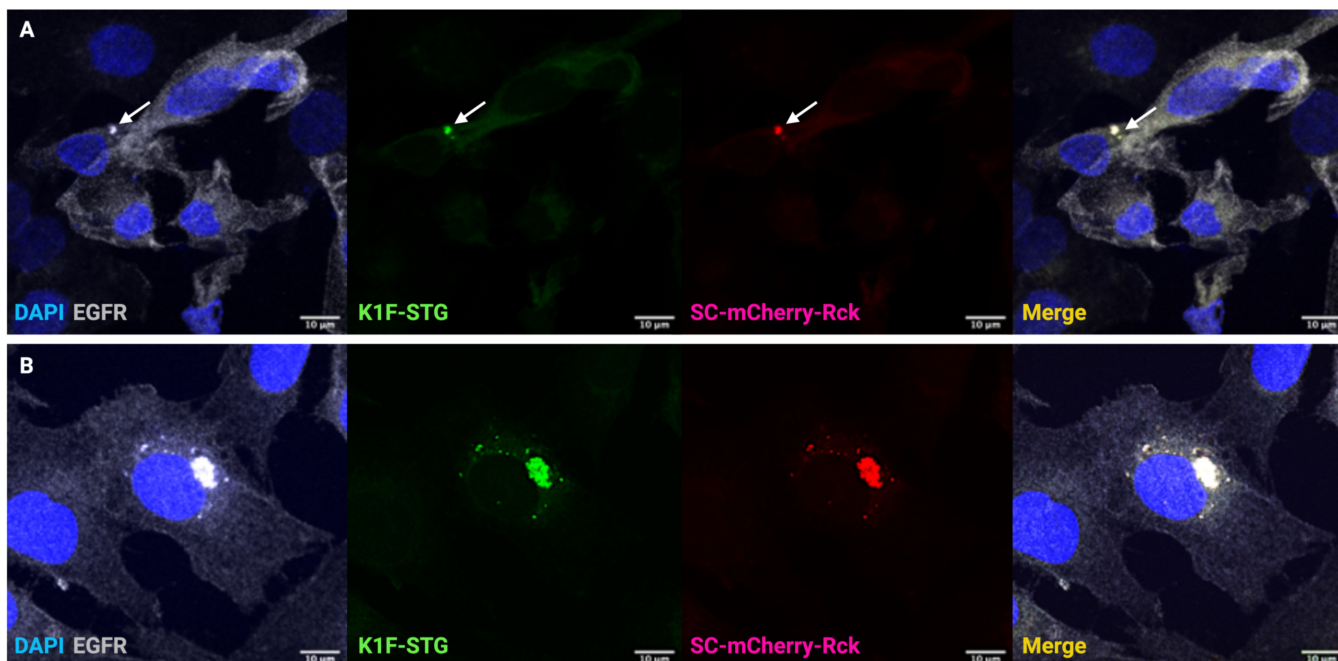

Figure 7: Altering mechanism of phage entry with SpyCatcher-mCherry-Rck capsid decoration. Immunocytochemistry fluorescence microscopy of cell-free TXTL assembled K1F-STG (green) decorated with SpyCatcher-mCherry-Rck (red), to enable activation of the EGF receptor (grey) in a T24 human bladder epithelial cell line stained with DAPI (blue). White arrows indicate interaction of Rck decorated phage with EGFR (A). Activation of EGFR leads to endocytic entry as demonstrated by the presence of endosomes in the cytosol (B).

commercially synthesised gene fragments, and used directly in linear template TXTL reactions, thereby avoiding laborious cloning and plasmid DNA template preparation steps (Figure S7).[46] Following reaction completion, the SpyCatcher fusion proteins were purified out of the TXTL reaction using small scale immobilised metal affinity chromatography (IMAC) purification. In parallel, the scaffold K1F-STG phage was expressed from its purified genome in separate TXTL reactions prior to purification using a 100 kDa MWCO filtration unit. The purified SpyCatcher fusion proteins and K1F-STG phage were combined to allow the SpyTag and SpyCatcher to form a covalent interaction. This covalent interaction results in the post-translational decoration of the capsid head of the phage with either SpyCatcher-mCherry-EGF or SpyCatcher-mCherry-Rck. In this manner, it was possible to generate two phenotypically distinct bacteriophage with the same underlying genotype, thereby proving the simplicity of this capsid modification method.

Following their creation, the EGF and Rck decorated phage were used to infect T24 human urinary bladder epithelial cells stained with a primary antibody for the EGF receptor. As stated previously, coupling between K1F-STG phage and the SpyCatcher-mCherry-[EGF/Rck] fusion was visible as punctate co-localised signals of GFP and mCherry respectively (Figure 6 and 7). The frequency of this co-localisation was much higher for the EGF decoration than for Rck. We attributed this to the higher levels of expression of EGF in our TXTL system compared to Rck. As such, when capsid decorations were performed, the SpyCatcher-mCherry-Rck protein concentration was not sufficient to saturate all available SpyTag proteins on the head of K1F-STG phage (Figure S8). Nonetheless, both phenotypes showed a strong affinity with the EGF receptor as seen by the localisation of the GFP and mCherry signals with the signal for the EGFR antibody at the periphery of human cells (Figure 6A and 7A). Subsequent activation of EGFR facilitated the endocytic entry of EGF/Rck decorated phage, evidenced

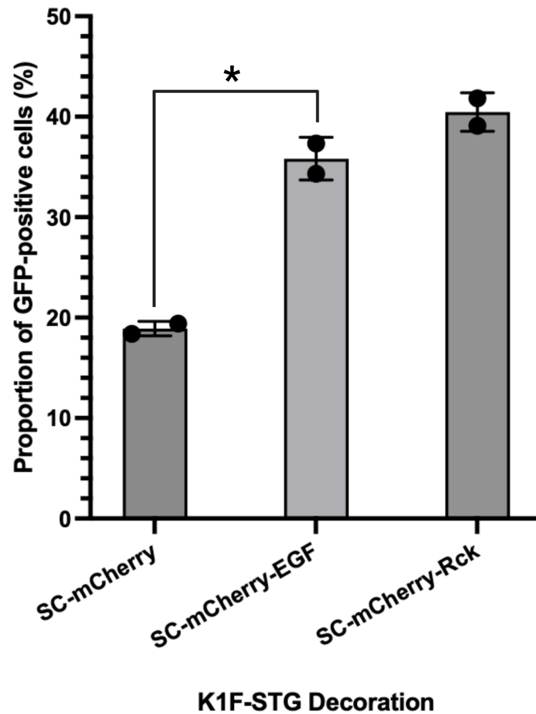

Figure 8: Quantitative analysis of decoration efficacy. Quantitative analysis of microscopy data was performed to determine the increase in phage entry into human cells when the decoration targets the phage to EGFR. To quantify the efficacy of the K1F derivatives, a total of 20 field-of-view images were taken, in which the efficacy was calculated as the total number of GFP and mCherry positive cells in comparison to the total number of cells. At least 200 cells were enumerated per condition. The error bars represent the standard deviation between two biological replicates, in which the data points are the average number of positive cells of each replicate. A two-tailed, paired T-test revealed a statistically significant difference ($P \leq 0.05$) between the SpyCatcher-mCherry and SpyCatcher-mCherry-EGF decorations (*). No significant difference was observed between SpyCatcher-mCherry-EGF and SpyCatcher-mCherry-Rck decorations.

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by the presence of phage-containing cytosolic endosomes inside human cells (Figure 6B and 7B). Furthermore, as EGF and Rck decorated phage are specifically targeted for endocytic entry, they enter human cells at much higher rates compared to phage decorated with SpyCatcher-mCherry alone (Figure 8). This quantitztive analysis of phage entry illustrate how the decoration of the phage with either EGF or Rck targets the phage to the EGFR resulting in 2-fold greater internalisation of phage compared to those decorated with SpyCatcher-mCherry alone. Once inside, EGF/Rck decorated phages are protected inside endosomes and would be able to persist for longer inside human cells (Table 1). It is also expected that the complement resistance effect imparted by the Rck decoration will further enhance the bioavailability, though further work is needed to quantify this phenomenon.

Importantly, TXTL assembled and capsid decorated phage produced using the above-described method had comparable endotoxin levels to undecorated CsCl purified K1F-STG phage (data not shown). The low endotoxin levels were also evidenced by the fact that the infected human cells did not elicit an immune response visible by microscopy. These levels were below the recommended levels for *in vivo* administration of phage thereby ensuring the applicability of this method for phage therapy.[33]

Table 1: *Summary of Microscopy Data*

Scaffold Phage	**Decoration**	**Observed Signals/New Phenotype**
K1F-WT	SpyCatcher-mCherry (40.3 kDa)	• No mCherry signal inside human cells • K1F-WT cannot be decorated due to lack of SpyTag • **No new phenotype**
K1F-GFP	SpyCatcher-mCherry (40.3 kDa)	• No mCherry signal inside human cells • K1F-GFP signal inside human cells • K1F-GFP does not interact with EGFR • K1F-GFP cannot be decorated due to lack of SpyTag • **No new phenotype**
K1F-STG (1)	SpyCatcher-mCherry (40.3 kDa)	• Co-localisation between mCherry and GFP due to covalent attachment of SpyCatcher-mCherry to K1F-STG and subsequent translocation into human cells. • mCherry decorated K1F-STG does not interact with EGFR • **New Phenotype: mCherry fluorescence**
K1F-STG (2)	SpyCatcher-mCherry-EGF (47.7 kDa)	• Co-localisation between mCherry and GFP due to covalent attachment of SpyCatcher-mCherry-EGF to K1F-STG and subsequent translocation into human cells • mCherry and GFP signals co-localised with anti-EGFR signal, demonstrating tropism to EGFR • Early endosomes containing phage inside human cells demonstrate endocytic entry • **New Phenotypes: mCherry fluorescence, EGF capsid decoration, endocytic entry and increased cellular uptake**
K1F-STG (3)	SpyCatcher-mCherry-Rck (56.2 kDa)	• Co-localisation between mCherry and GFP due to covalent attachment of SpyCatcher-mCherry-Rck to K1F-STG and subsequent translocation into human cells • mCherry and GFP signals co-localised with anti-EGFR signal, demonstrating tropism to EGFR • Early endosomes containing phage inside human cells demonstrate endocytic entry • **New Phenotypes: mCherry fluorescence, Rck capsid decoration, endocytic entry, and increased cellular uptake**

Conclusions and Future Directions

The proposed strategy thus aims to streamline the research workflow to enable rapid screening of multiple protein candidates through post-translational decoration of a single engineered phage using a cell-free TXTL system, in less than two working days. It may be postulated that a limitation of the methods described in this work is the loss of the capsid decoration after a single infection event. However, in the context of phage therapy, a transient capsid decoration could still serve to help the initially administered phage to evade the human immune system, enter target cells via a preferred receptor-mediated pathway or provide an auxiliary antimicrobial mechanism to the phage itself.

Taking inspiration from advances in cancer immunotherapy, decoration strategies could also enable the creation of personalised phage therapies whereby SpyTagged phage cocktails are decorated with patient-specific proteins that are designed to suppress an immune response against the phage, thus providing a simple solution for enabling the re-administration and reuse of specific phage cocktails (Figure 9).[47–50]More broadly, the impact of the SpyPhage platform could also benefit applications of engineered phage beyond their simple use as antimicrobial agents. Recent efforts have utilised phage for a myriad of different functions from tumor targeting bacteriophages, vectors for gene therapy and rapid vaccine development.[51] The real world impact of all such applications could be sped up by simplifying the engineering pipeline through the use of a single engineered scaffold phage decorated with a desired post-translational capsid modification and may allow researchers to overcome regulatory obstacles currently holding back the clinical use of bacteriophages.[52]

Despite the promise of the SpyPhage platform, its incorporation into a future phage therapy production pipeline would require a number of technical challenges to be met. Firstly, future research efforts must focus on developing methods that omit the use of hazardous chemicals such as chloroform in the preparation of phage therapies.[53] Chloroform was used in our method to denature crude extract proteins following assembly of K1F-STG phage in a CFR. As K1F is chloroform resistant, this method was a simple solution to purify the phage from the TXTL reaction. Nonetheless, the capsid decoration method described does also allow for direct SpyTag/SpyCatcher based purification of TXTL assembled phage using Ni-NTA magnetic beads pre-loaded with His_6-tagged SpyCatcher fusion protein (Supporting Information S9). However, we found the direct purification method led to poorly decorated phage as observed by lower co-localised signal intensities visible under microscopy (data not shown). As such, direct affinity based purification strategies of capsid decorated phage from CFRs need further refinement and development in the coming years.

Next we foresee the need to engineer the next generation of cell-free TXTL systems geared solely towards the production of bacteriophages for phage therapy. The development of a minimal CFS, potentially through the rational re-engineering of the PURE TXTL system to enable phage production, would not only serve to overcome regulatory hurdles but also aid in illuminating a complete bottom-up mechanistic understanding of phage replication *in vitro*.[54]Such systems would function as heterologous expression platforms, containing phage specific transcription factors, that would enable the production of a variety of different phage within a single minimal CFS derived from a non-pathogenic host (Figure 9B).[55] Further still, achieving concurrent whole genome assembly of an engineered genome within a minimal CFS capable of expressing virulent phage particles from the newly assembled genome, is a significant milestone yet to be reached.[56–58]These strategies would forego the need for cultivation of pathogenic bacterial hosts in a future phage engineering pipeline.

Finally, the cost-effective and rapid production of future phage therapies will require centralised infrastructure pertaining to the academic understanding of phages, their clinical use and methods for their engineering into effective therapies.[59] Key to this will be the creation of phage bio-foundries that aim to

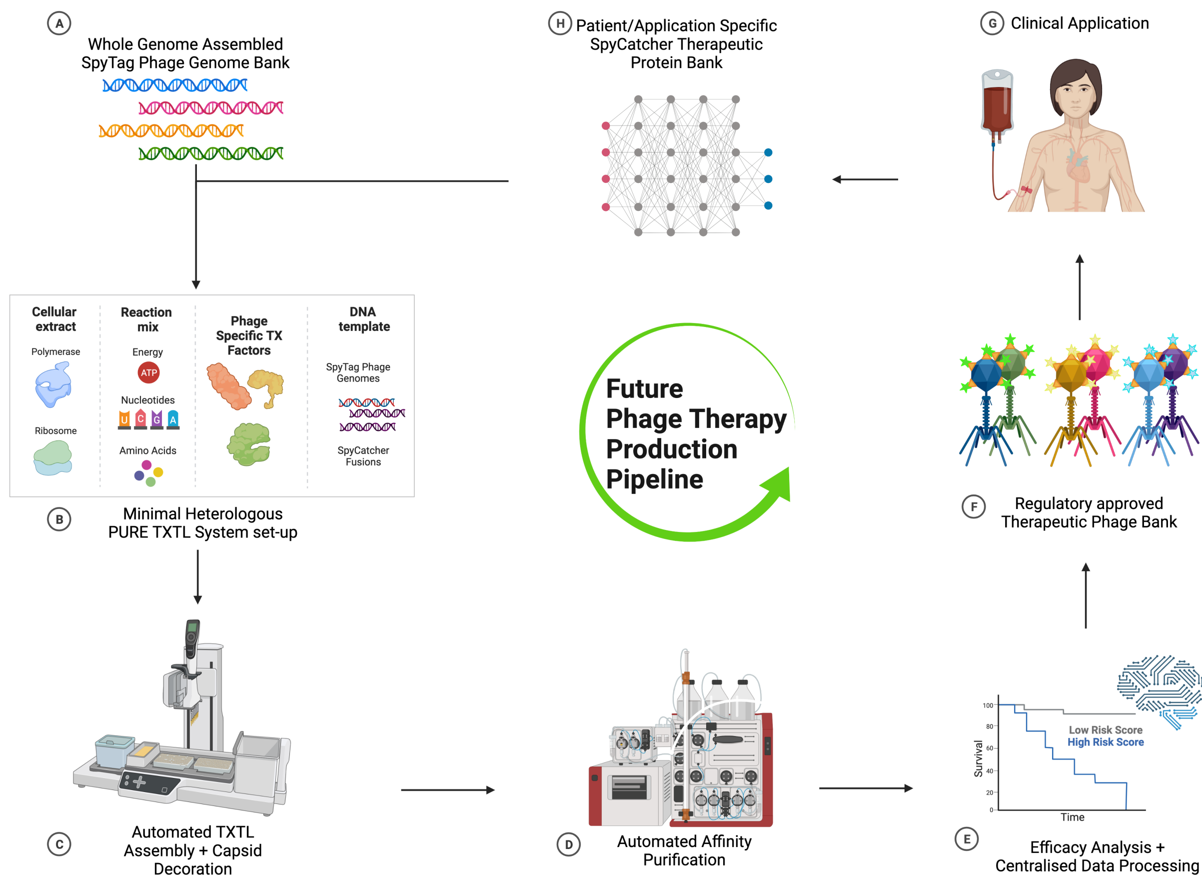

Figure 9: Future phage therapy production pipeline. Whole genome assembled SpyTag phage genome bank and automated expression and purification of SpyTag phage in a heterologous PURE TXTL system will eliminate the use of pathogenic bacteria (A-D). Efficacy testing and centralised data collection through phage foundry networks will help create patient and application-specific phage therapies in the near future (E-H).

fill the knowledge and technological gaps in phage therapeutics.[60] By combining such co-ordinated efforts with cutting-edge synthetic biology, rational therapeutic design and automation, generally applicable tools for rapid phage engineering can be built to enable the next generation of phage therapies.

3 Materials and Methods

CRISPR/Cas9-based Engineering of K1F

K1F-gene10b::GFP-SpyTag (K1F-STG) phage was engineered using plasmid based homologous recombination, facilitated by CRISPR/Cas9 selection, as described previously.[15] Briefly, K1F-STG was engineered by growing the K1F-GFP phage (carrying g10b::GFP) on *E. coli* EV36/pUC-GFP-ST. The resulting phage mix was enriched on *E. coli* EV36 + pCas9GFP-ST for three rounds, followed by a plaque assay and plaque PCR to screen for engineered phage. The engineered segments of the phage were PCR-amplified for verification by Sanger sequencing. Donor cassette design and sequence of pUC-GFP-ST is provided in Supporting Information Figure S2 and Table S3 respectively. Detailed protocol is provided in Supporting Information S2.

Whole Genome Assembly

As an alternative approach to the CRISPR-based phage engineering method, K1F-gene10b::SpyTag (K1F-STB) was created through whole genome assembly. Briefly, the K1F genome was split into 8 fragments with approximately 40bp of homology between adjacent fragments. All fragments (except fragment 5) were PCR amplified using Phusion

Polymerase (NEB) from the wild-type genome and gel extracted, while fragment 5 was commercially synthesised (IDT) to code for SpyTag insertion into gene10b, and used directly in the Gibson assembly. Fragment 5 sequences for SpyTag fusion to gene10b can be found in Table S4. Whole genome Gibson assembly was performed using the GeneArt Gibson Assembly EX System (Invitrogen) and ethanol precipitated prior to transformation into DH5a cells (Invitrogen). Following overgrowth of the transformation, cells were lysed with chloroform to release entrapped phage. Lysates were plaque assayed and SpyTag insertion was confirmed via PCR as previously described. Detailed whole genome assembly protocol can be found in Supporting Information S3.

Crude Extract Preparation

Crude extract was prepared by growing *E. coli* Rosetta 2 Cells (Novagen) harbouring pAD-LyseR plasmid (Addgene #99244) at 37°C until to OD_{600} of 1.8. Cells were pelleted and washed in S30A buffer and frozen at -80°C. Cell pellets were thawed to room temperature to facilitate lysis via lambda endolysin gene R expressed by the pAD-LyseR plasmid during growth. Thawed cells were subsequently treated with lysozyme and sonicated before proceeding to post-lysis processing as described in Supporting Information S4.

Cell-Free TXTL Phage Assembly and Capsid Decoration

All cell-free TXTL reactions used in this study had a volume of 20 μL and contained additive components at concentrations as detailed in Supplementary Information S7. Linear DNA templates for the expression of fusion proteins in TXTL were PCR amplified from synthesised gene fragments using Phusion polymerase (NEB) and PCR purified using Monarch DNA Clean up Kit (NEB). For a typical capsid decoration experiment, independent TXTL reactions expressing a K1F phage variants or capsid decoration proteins were set up and incubated for 5 hours at 29°C. Capsid decoration proteins were purified from TXTL reactions by IMAC using NEBExpress Ni-NTA Magnetic Beads (NEB). Capsid decorations of phage were performed by combining 100 μL of decorative protein in PBS with an equal volume of TXTL assembled and purified phage in PBS and incubated at 29°C for 2 hours to allow for coupling of SpyCatcher fused decoration protein to SpyTag on phage head. A 100 kDa MWCO filtration unit (Millipore) was used to remove any uncoupled fusion protein and to concentrate the capsid decorated phage into a final volume of 100 μL. Following their preparation, capsid decorated phage were used immediately in downstream human cell infection experiments. Detailed protocols for purification from TXTL reactions and capsid decoration can be found in Supporting Information S8 and S9.

Immunocytochemistry and Fluorescence Microscopy

Immunocytochemistry and fluorescence microscopy was undertaken in order to visualise the association between the K1F-STG and SpyCatcher-mCherry fusions that were post-translationally coupled to the capsid head of the phage. Typically, 10^7 PFU/mL of capsid decorated phage were added to T24 human bladder epithelial cells and incubated for 1 hour. Capsid decoration was visualised within human cells as the co-localisation of GFP and mCherry signals from the phage and capsid decoration protein respectively. The ability of EGF/Rck capsid decoration to alter the mechanism of phage entry into human cells was visualised as the co-localisation of the GFP and mCherry signal with the EGF receptor, as stained for by an EGFR antibody. Detailed experimental procedures are provided in Supporting Information S10.

4 Author Information

Author Contributions:

S.B.W.L. conceived the project, executed experiments and prepared manuscript. J.W. performed immunocytochemistry and fluorescence microscopy imaging. J.P.W. assisted in cell-free system set-up. S.B.W.L. created phage K1F-STB *via* whole genome assembly and executed the cell-free TXTL capsid decoration pipeline.

S.B.W.L. designed plasmids for the creation of phage K1F-STG. A.Y.B. and T.F. created phage K1F-STG. M.H. assisted in protein expression and purification. Phage K1F-WT, phage K1F-GFP, host strain *E. coli* EV36, T24 human bladder epithelial cells and reagents for microscopy were provided by A.P.S. Manuscript was revised by K.J.P., R.J.P., T.F. and V.K.

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References

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(1) Gordillo Altamirano, F. L.; Barr, J. J. Unlocking the next generation of phage therapy: the key is in the receptors. *Curr Opin Biotechnol* **2021**, *68*, 115-123. DOI: 10.1016/j.copbio.2020.10.002.

(2) Rosner, D.; Clark, J. Formulations for Bacteriophage Therapy and the Potential Uses of Immobilization. *Pharmaceuticals (Basel)* **2021**, *14* (4). DOI: 10.3390/ph14040359.

(3) Zhang, D.; Bi, H.; Liu, B.; Qiao, L. Detection of Pathogenic Microorganisms by Microfluidics Based Analytical Methods. *Anal Chem* **2018**, *90* (9), 5512-5520. DOI: 10.1021/acs.analchem.8b00399.

(4) Brives, C.; Pourraz, J. Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. *Palgrave Communications* **2020**, *6* (1). DOI: 10.1057/s41599-020-0478-4.

(5) Batinovic, S.; Wassef, F.; Knowler, S. A.; Rice, D. T. F.; Stanton, C. R.; Rose, J.; Tucci, J.; Nittami, T.; Vinh, A.; Drummond, G. R.; et al. Bacteriophages in Natural and Artificial Environments. *Pathogens* **2019**, *8* (3). DOI: 10.3390/pathogens8030100.

(6) Furfaro, L. L.; Payne, M. S.; Chang, B. J. Bacteriophage Therapy: Clinical Trials and Regulatory Hurdles. *Front Cell Infect Microbiol* **2018**, *8*, 376. DOI: 10.3389/fcimb.2018.00376.

(7) Gorski, A.; Borysowski, J.; Miedzybrodzki, R. Phage Therapy: Towards a Successful Clinical Trial. *Antibiotics (Basel)* **2020**, *9* (11). DOI: 10.3390/antibiotics9110827.

(8) Zaczek, M.; Weber-Dabrowska, B.; Miedzybrodzki, R.; Lusiak-Szelachowska, M.; Gorski, A. Phage Therapy in Poland - a Centennial Journey to the First Ethically Approved Treatment Facility in Europe. *Front Microbiol* **2020**, *11*, 1056. DOI: 10.3389/fmicb.2020.01056.

(9) Barbu, E. M.; Cady, K. C.; Hubby, B. Phage Therapy in the Era of Synthetic Biology. *Cold Spring Harb Perspect Biol* **2016**, *8* (10). DOI: 10.1101/cshperspect.a023879.

(10) Hodgman, C. E.; Jewett, M. C. Cell-free synthetic biology: thinking outside the cell. *Metab Eng* **2012**, *14* (3), 261-269. DOI: 10.1016/j.ymben.2011.09.002.

(11) Shin, J.; Jardine, P.; Noireaux, V. Genome replication, synthesis, and assembly of the bacteriophage T7 in a single cell-free reaction. *ACS Synth Biol* **2012**, *1* (9), 408-413. DOI: 10.1021/sb300049p.

(12) Chen, Y.; Batra, H.; Dong, J.; Chen, C.; Rao, V. B.; Tao, P. Genetic Engineering of Bacteriophages Against Infectious Diseases. *Front Microbiol* **2019**, *10*, 954. DOI:

10.3389/fmicb.2019.00954.

(13) Kilcher, S.; Studer, P.; Muessner, C.; Klumpp, J.; Loessner, M. J. Cross-genus rebooting of custom-made, synthetic bacteriophage genomes in L-form bacteria. *Proc Natl Acad Sci U S A* **2018**, *115* (3), 567-572. DOI: 10.1073/pnas.1714658115.

(14) Garamella, J.; Marshall, R.; Rustad, M.; Noireaux, V. The All E-coli TX-TL Toolbox 2.0: A Platform for Cell-Free Synthetic Biology. *Acs Synthetic Biology* **2016**, *5* (4), 344-355. DOI: 10.1021/acssynbio.5b00296.

(15) Rustad, M.; Eastlund, A.; Jardine, P.; Noireaux, V. Cell-free TXTL synthesis of infectious bacteriophage T4 in a single test tube reaction. *Synth Biol (Oxf)* **2018**, *3* (1), ysy002. DOI: 10.1093/synbio/ysy002.

(16) Garenne, D.; Thompson, S.; Brisson, A.; Khakimzhan, A.; Noireaux, V. The all-E. coliTXTL toolbox 3.0: new capabilities of a cell-free synthetic biology platform. *Synth Biol (Oxf)* **2021**, *6* (1), ysab017. DOI: 10.1093/synbio/ysab017.

(17) Krishnan, S.; Chang, A. C.; Stoltz, B. M.; Prasadarao, N. V. Escherichia coli K1 Modulates Peroxisome Proliferator-Activated Receptor gamma and Glucose Transporter 1 at the Blood-Brain Barrier in Neonatal Meningitis. *J Infect Dis* **2016**, *214* (7), 1092-1104. DOI: 10.1093/infdis/jiw306.

(18) Gordon, S. M.; Srinivasan, L.; Harris, M. C. Neonatal Meningitis: Overcoming Challenges in Diagnosis, Prognosis, and Treatment with Omics. *Front Pediatr* **2017**, *5*, 139. DOI: 10.3389/fped.2017.00139.

(19) Moller-Olsen, C.; Ho, S. F. S.; Shukla, R. D.; Feher, T.; Sagona, A. P. Engineered K1F bacteriophages kill intracellular Escherichia coli K1 in human epithelial cells. *Sci Rep* **2018**, *8* (1), 17559. DOI: 10.1038/s41598-018-35859-6.

(20) Gorski, A.; Jonczyk-Matysiak, E.; Lusiak-Szelachowska, M.; Miedzybrodzki, R.; Weber-Dabrowska, B.; Borysowski, J. The Potential of Phage Therapy in Sepsis. *Front Immunol* **2017**, *8*, 1783. DOI: 10.3389/fimmu.2017.01783.

(21) Zakeri, B.; Fierer, J. O.; Celik, E.; Chittock, E. C.; Schwarz-Linek, U.; Moy, V. T.; Howarth, M. Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proc Natl Acad Sci U S A* **2012**, *109* (12), E690-697. DOI: 10.1073/pnas.1115485109.

(22) Brune, K. D.; Leneghan, D. B.; Brian, I. J.; Ishizuka, A. S.; Bachmann, M. F.; Draper, S. J.; Biswas, S.; Howarth, M. Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization. *Sci Rep* **2016**, *6*, 19234. DOI: 10.1038/srep19234.

(23) Pulkkinen, E. M.; Hinkley, T. C.; Nugen, S. R. Utilizing in vitro DNA assembly to engineer a synthetic T7 Nanoluc reporter phage for Escherichia coli detection. *Integr Biol (Camb)* **2019**. DOI: 10.1093/intbio/zyz005.

(24) Zainabadi, K.; Dhayabaran, V.; Moideen, K.; Krishnaswamy, P. An efficient and cost-effective method for purification of small sized DNAs and RNAs from human urine. *PLoS One* **2019**, *14* (2), e0210813. DOI: 10.1371/journal.pone.0210813.

(25) Carroll-Portillo, A.; Coffman, C. N.; Varga, M. G.; Alcock, J.; Singh, S. B.; Lin, H. C. Standard Bacteriophage Purification Procedures Cause Loss in Numbers and Activity. *Viruses* **2021**, *13* (2). DOI: 10.3390/v13020328.

(26) Siegal-Gaskins, D.; Tuza, Z. A.; Kim, J.; Noireaux, V.; Murray, R. M. Gene circuit performance characterization and resource usage in a cell-free "breadboard". *ACS Synth Biol* **2014**, *3* (6), 416-425. DOI: 10.1021/sb400203p.

(27) Scholl, D.; Merril, C. The genome of bacteriophage K1F, a T7-like phage that has acquired the ability to replicate on K1 strains of Escherichia coli. *J Bacteriol* **2005**, *187* (24), 8499-8503. DOI: 10.1128/JB.187.24.8499-8503.2005.

(28) Sun, Z. Z.; Hayes, C. A.; Shin, J.; Caschera, F.; Murray, R. M.; Noireaux, V. Protocols for implementing an Escherichia coli based TX-TL cell-free expression system for synthetic biology. *J Vis Exp* **2013**, (79), e50762. DOI: 10.3791/50762.

(29) Didovyk, A.; Tonooka, T.; Tsimring, L.; Hasty, J. Rapid and Scalable Preparation of Bacterial Lysates for Cell-Free Gene Expression. *ACS Synth Biol* **2017**, *6* (12), 2198-2208. DOI: 10.1021/acssynbio.7b00253.

(30) Rustad, M.; Eastlund, A.; Marshall, R.; Jardine, P.; Noireaux, V. Synthesis of Infectious Bacteriophages in an E. coli-based Cell-free Expression System. *J Vis Exp* **2017**, (126). DOI: 10.3791/56144.

(31) Falgenhauer, E.; von Schonberg, S.; Meng, C.; Muckl, A.; Vogele, K.; Emslander, Q.; Ludwig, C.; Simmel, F. C. Evaluation of an E. coli Cell Extract Prepared by Lysozyme-Assisted Sonication via Gene Expression, Phage Assembly and Proteomics. *Chembiochem* **2021**. DOI: 10.1002/cbic.202100257.

(32) Sun, Z. Z.; Yeung, E.; Hayes, C. A.; Noireaux, V.; Murray, R. M. Linear DNA for Rapid Prototyping of Synthetic Biological Circuits in an Escherichia coli Based TX-TL Cell-Free System. *ACS Synthetic Biology* **2014**, *3* (6), 387-397. DOI: 10.1021/sb400131a.

(33) Hietala, V.; Horsma-Heikkinen, J.; Carron, A.; Skurnik, M.; Kiljunen, S. The Removal of Endo- and Enterotoxins From Bacteriophage Preparations. *Front Microbiol* **2019**, *10*, 1674. DOI: 10.3389/fmicb.2019.01674.

(34) Li, L.; Fierer, J. O.; Rapoport, T. A.; Howarth, M. Structural analysis and optimization of the covalent association between SpyCatcher and a peptide Tag. *J Mol Biol* **2014**, *426* (2), 309-317. DOI: 10.1016/j.jmb.2013.10.021.

(35) Kassner, P. D.; Burg, M. A.; Baird, A.; Larocca, D. Genetic selection of phage engineered for receptor-mediated gene transfer to mammalian cells. *Biochem Biophys Res Commun* **1999**, *264* (3), 921-928. DOI: 10.1006/bbrc.1999.1603.

(36) Larocca, D.; Jensen-Pergakes, K.; Burg, M. A.; Baird, A. Receptor-targeted gene delivery using multivalent phagemid particles. *Mol Ther* **2001**, *3* (4), 476-484. DOI: 10.1006/mthe.2001.0284.

(37) Dabrowska, K. Phage therapy: What factors shape phage pharmacokinetics and bioavailability? Systematic and critical review. *Med Res Rev* **2019**, *39* (5), 2000-2025. DOI: 10.1002/med.21572.

(38) Lemmon, M. A.; Schlessinger, J.; Ferguson, K. M. The EGFR family: not so prototypical receptor tyrosine kinases. *Cold Spring Harb Perspect Biol* **2014**, *6* (4), a020768. DOI: 10.1101/cshperspect.a020768.

(39) Zhang, X.; Gureasko, J.; Shen, K.; Cole, P. A.; Kuriyan, J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* **2006**, *125* (6), 1137-1149. DOI: 10.1016/j.cell.2006.05.013.

(40) Mambu, J.; Virlogeux-Payant, I.; Holbert, S.; Grepinet, O.; Velge, P.; Wiedemann, A. An Updated View on the Rck Invasin of Salmonella: Still Much to Discover. *Front Cell Infect Microbiol* **2017**, *7*, 500. DOI: 10.3389/fcimb.2017.00500.

(41) Rosselin, M.; Virlogeux-Payant, I.; Roy, C.; Bottreau, E.; Sizaret, P. Y.; Mijouin, L.; Germon, P.; Caron, E.; Velge, P.; Wiedemann, A. Rck of Salmonella enterica, subspecies enterica serovar enteritidis, mediates zipper-like internalization. *Cell Res* **2010**, *20* (6), 647-664. DOI: 10.1038/cr.2010.45.

(42) Ho, D. K.; Jarva, H.; Meri, S. Human complement factor H binds to outer membrane protein Rck of Salmonella. *J Immunol* **2010**, *185* (3), 1763-1769. DOI: 10.4049/jimmunol.1001244.

(43) Ho, D. K.; Tissari, J.; Jarvinen, H. M.; Blom, A. M.; Meri, S.; Jarva, H. Functional recruitment of human complement inhibitor C4B-binding protein to outer membrane protein Rck of Salmonella. *PLoS One* **2011**, *6* (11), e27546. DOI: 10.1371/journal.pone.0027546.

(44) Koczerka, M.; Douarre, P. E.; Kempf, F.; Holbert, S.; Mistou, M. Y.; Grepinet, O.; Virlogeux-Payant, I. The Invasin and Complement-Resistance Protein Rck of Salmonella is More Widely Distributed than Previously Expected. *Microbiol Spectr* **2021**, *9* (2), e0145721. DOI: 10.1128/Spectrum.01457-21.

(45) Reddington, S. C.; Howarth, M. Secrets of a covalent interaction for biomaterials and biotechnology: SpyTag and SpyCatcher. *Curr Opin Chem Biol* **2015**, *29*, 94-99. DOI: 10.1016/j.cbpa.2015.10.002.

(46) Yim, S. S.; Johns, N. I.; Noireaux, V.; Wang, H. H. Protecting Linear DNA Templates in Cell-Free Expression Systems from Diverse Bacteria. *ACS Synth Biol* **2020**, *9* (10), 2851-2855. DOI: 10.1021/acssynbio.0c00277.

(47) Goracci, M.; Pignochino, Y.; Marchio, S. Phage Display-Based Nanotechnology Applications in Cancer Immunotherapy. *Molecules* **2020**, *25* (4). DOI: 10.3390/molecules25040843.

(48) Hess, K. L.; Jewell, C. M. Phage display as a tool for vaccine and immunotherapy development. *Bioeng Transl Med* **2020**, *5* (1), e10142. DOI: 10.1002/btm2.10142.

(49) Waldman, A. D.; Fritz, J. M.; Lenardo, M. J. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* **2020**, *20* (11), 651-668. DOI: 10.1038/s41577-020-0306-5.

(50) Kabwe, M.; Dashper, S.; Bachrach, G.; Tucci, J. Bacteriophage manipulation of the microbiome associated with tumour microenvironments-can this improve cancer therapeutic response? *FEMS Microbiol Rev* **2021**, *45* (5). DOI: 10.1093/femsre/fuab017.

(51) Gibb, B.; Hyman, P.; Schneider, C. L. The Many Applications of Engineered Bacteriophages—An Overview. *Pharmaceuticals* **2021**, *14* (7), 634. DOI: 10.3390/ph14070634

(52) Reindel, R.; Fiore, C. R. Phage Therapy: Considerations and Challenges for Development. *Clin Infect Dis* **2017**, *64* (11), 1589-1590. DOI: 10.1093/cid/cix188.

(53) Liu, D.; Van Belleghem, J. D.; de Vries, C. R.; Burgener, E.; Chen, Q.; Manasherob, R.; Aronson, J. R.; Amanatullah, D. F.; Tamma, P. D.; Suh, G. A. The Safety and Toxicity of Phage Therapy: A Review of Animal and Clinical Studies. *Viruses* **2021**, *13* (7). DOI: 10.3390/v13071268.

(54) Lavickova, B.; Maerkl, S. J. A simple, robust, and low-cost method to produce the PURE cell-free system. *ACS Synth Biol* **2019**, *8* (2), 455-462. DOI: 10.1021/acssynbio.8b00427

(55) Emslander, Q.; Vogele, K.; Braun, P.; Stender, J.; Joppich, M.; Abele, M.; Meng, C.; Ludwig, C.; Bugert, J. J.; Willy, C. Personalized Production, Non-Genomic Engineering, and Time-Resolved Proteomics of Therapeutic Phages for Biohazardous and Multidrug-Resistant Bacteria. *Non-Genomic Engineering, and Time-Resolved Proteomics of Therapeutic Phages for Biohazardous and Multidrug-Resistant Bacteria.* DOI: 10.2139/ssrn.3887804

(56) Feher, T.; Karcagi, I.; Blattner, F. R.; Posfai, G. Bacteriophage recombineering in the lytic state using the lambda red recombinases. *Microb Biotechnol* **2012**, *5* (4), 466-476. DOI: 10.1111/j.1751-7915.2011.00292.x.

(57) Zhang, Y.; Werling, U.; Edelmann, W. SLiCE: a novel bacterial cell extract-based DNA cloning method. *Nucleic Acids Res* **2012**, *40* (8), e55. DOI: 10.1093/nar/gkr1288.

(58) Wurstle, S.; Stender, J.; Hammerl, J. A.; Vogele, K.; Rothe, K.; Willy, C.; Bugert, J. J. Practical Assessment of an Interdisciplinary Bacteriophage Delivery Pipeline for Personalized Therapy of Gram-Negative Bacterial Infections. *Pharmaceuticals (Basel)* **2022**, *15* (2). DOI: 10.3390/ph15020186.

(59) Suh, G. A.; Lodise, T. P.; Tamma, P. D.; Knisely, J. M.; Alexander, J.; Aslam, S.; Barton, K. D.; Bizzell, E.; Totten, K. M.; Campbell, J. Considerations for the Use of Phage Therapy in Clinical Practice. *Antimicrobial agents and chemotherapy* **2022**, AAC. 02071-02021. DOI: 10.1128/aac.02071-21

(60) Mutalik, V. K.; Arkin, A. P. A Phage Foundry framework to systematically develop viral countermeasures to combat antibiotic resistant bacterial pathogens. *Iscience* **2022**, 104121. DOI: 10.1016/j.isci.2022.104121