DISS. ETH NO. 29062

Synthetic Biology for Genetically Engineered Bacteriophages to Target Infectious Diseases

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2023

Abstract

The widespread, global availability and use of antibiotics has lead to a drastic increase in the number of antimicrobial resistant pathogens. The "golden age of antibiotics" from which humans have prospered for almost a century is in jeopardy. To avoid falling back into an era where even small infections can spread uncontrollably and lead to severe illness or death, there is an urgent need for alternative antimicrobials.

Bacterial viruses, also termed bacteriophages, or simply phages, are vastly abundant in nature and pose an ideal alternative due to their ability to effectively infect and kill their bacterial hosts. Although naturally occuring phages have been successfully employed as therapeutics, genetically engineered phages have received increased attention in recent years, as many pitfalls associated with naturally occurring phages can be overcome, thereby increasing their potential for clinical use.

In **Manuscript I**, we review state-of-the-art bacteriophage engineering methodologies, applications thereof, and discuss the outlook into the future of phage therapy, particularly in the context of machine learning-driven computational methods.

Staphylococcus aureus is a major human pathogen and, due to emergence of numerous strains exhibiting antibiotic resistance, even to last-line treatment options, novel antimicrobials are urgently needed. Although most suitable for phage therapy, the genetic engineering of large, lytic *S. aureus* phages with broad host range has so far proved difficult using conventional engineering methods. In **Manuscript II**, we use an homologous recombination-based and CRISPR Cas9 counterselection-assisted approach to engineer the large, lytic, *S. aureus*-infecting *Kayvirus* K. We used this novel method to integrate a bioluminescent reporter payload into the phage genome, thereby facilitating rapid detection of a large range of *S. aureus* strains, including a selection of vancomycin resistant

clinical isolates. Our engineered phage proved highly efficient both *in vitro* as well as in complex matrices such as human whole blood and bovine raw milk.

As mentioned previously, one conventional method of genetically engineering phages is using a synthetic biology approach including assembly of synthetic phage genome fragments and subsequent reactivation in a suitable host organism. This can prove challenging, particularly for Gram-positive species such as *S. aureus*, where introduced exogenous DNA is met by wide array of intracellular defense mechanisms. In **Manuscript III**, we describe a detailed protocol for the development of cell-wall deficient L-form bacteria, which are capable of taking up large DNA molecules via simple chemical transformation. We demonstrate the conversion to the L-form state for three different Gram-positive species, *Listeria monocytogenes*, *S. aureus* and *Staphylococcus xylosus*, and demonstrate their ability to reactivate various different phage genomes, even across the genus barrier.

Computational methods are already an essential part of modern science and likely will continue to be an integral component for driving forward advances in biological research, particularly in genetic engineering. In **Manuscript IV** we developed a machine learning-based method for predicting transcriptional promoters from primary phage sequence data. We used the same bioluminescent payload from **Manuscript II**, to experimentally validate our approach. We demonstrate that the accurate prediction of payload expression levels, based on the location of insertion sites behind promoters with various predicted expression strengths, is indeed possible. This has great potential for future applications to control the expression levels of effector payloads integrated into the phage backbone.

One approach for enhancing phage efficacy for therapeutic applications is the genetic arming with antimicrobial effector payloads. In **Manuscript V**, we screened a large selection of payload gene candidates, which had previously been shown to exhibit bactericidal activity against *S. aureus*, for their suitability as effector payloads in K. Although we failed to obtain viable phages for the majority of payload genes, we succeeded in devel-

oping two engineered phages containing the protein toxin MazF and the short-leaderless bacteriocin Lacticin Q as a genetic payload, respectively. Both phages showed significantly enhanced *in vitro* killing efficiency on a selection of *S. aureus* hosts when compared to the wildtype phage.

With this thesis, I hope to have brought insight and advances into the field of phage engineering, specifically in the context of *S. aureus*-targeted applications. By demonstrating different approaches based on phage type and intended modification, we show that phage engineering has great potential for developing modified phages with enhanced properties directed towards future therapeutic applications. Overall, phage engineering and therapy shows great promise to mitigate the effects of the globally emerging antibiotic resistance crisis, and has the potential to be a major therapeutic in future times.

Zusammenfassung

Die weit verbreitete, weltweite Verfügbarkeit und Verwendung von Antibiotika hat zu einem drastischen Anstieg der Zahl antimikrobiell resistenter Krankheitserreger geführt. Das "goldene Zeitalter der Antibiotika", von dem die Menschen fast ein Jahrhundert lang profitiert haben, ist in Gefahr. Um einen Rückfall in eine Ära zu vermeiden, in der sich selbst kleine Infektionen unkontrolliert ausbreiten und zu schweren Erkrankungen oder zum Tod führen können, besteht dringender Bedarf an alternativen antimikrobiellen Mitteln.

Bakterielle Viren, auch Bakteriophagen oder einfach Phagen genannt, kommen in der Natur in großer Zahl vor und stellen aufgrund ihrer Fähigkeit, ihre bakteriellen Wirte wirksam zu infizieren und abzutöten, eine ideale Alternative dar. Obwohl natürlich vorkommende Phagen bereits erfolgreich als Therapeutika eingesetzt wurden, haben gentechnisch hergestellte Phagen in den letzten Jahren zunehmend an Aufmerksamkeit gewonnen, da viele Probleme, die mit natürlich vorkommenden Phagen verbunden sind, überwunden werden können, was ihr Potenzial für den klinischen Einsatz erhöht.

In **Manuskript I** geben wir einen Überblick über den Stand der Technik bei der Entwicklung von Bakteriophagen und deren Anwendungen und diskutieren einen Ausblick auf die Zukunft der Phagentherapie, insbesondere im Zusammenhang mit maschinellem Lernen.

Staphylococcus aureus ist ein wichtiger Krankheitserreger des Menschen, und aufgrund des Auftretens zahlreicher Stämme, die eine Antibiotikaresistenz aufweisen, selbst gegen die letzten Behandlungsmöglichkeiten, werden dringend neue Antimikrobiotika benötigt. Obwohl sie für die Phagentherapie bestens geeignet sind, hat sich die gentechnische Herstellung großer, lytischer *S. aureus*-Phagen mit breitem Wirtsspektrum

mit herkömmlichen Methoden bisher als schwierig erwiesen. In **Manuskript II** verwenden wir einen auf homologer Rekombination basierenden und durch CRISPR Cas9 unterstützten Ansatz, um den großen, lytischen, *S. aureus*-infizierenden *Kayvirus* K zu entwickeln. Wir haben diese neuartige Methode verwendet, um eine biolumineszente Reporter-Nutzlast in das Phagengenom zu integrieren und dadurch eine schnelle Erkennung einer Vielzahl von *S. aureus*-Stämmen zu ermöglichen, einschließlich einer Auswahl von Vancomycin-resistenten klinischen Isolaten. Die von uns entwickelten Phagen erwiesen sich sowohl *in vitro* als auch in komplexen Matrizen wie menschlichem Vollblut und Rinderrohmilch als äußerst effizient.

Wie bereits erwähnt, besteht eine herkömmliche Methode zur gentechnischen Veränderung von Phagen in der Verwendung eines synthetischen biologischen Ansatzes, der die Zusammenstellung synthetischer Phagengenomfragmente und die anschließende Reaktivierung in einem geeigneten Wirtsorganismus umfasst. Dies kann sich als Herausforderung erweisen, insbesondere bei Gram-positiven Arten wie *S. aureus*, wo die eingebrachte exogene DNA auf eine Vielzahl von intrazellulären Abwehrmechanismen trifft. In **Manuskript III** beschreiben wir ein detailliertes Protokoll für die Entwicklung von L-Form Bakterien, welche keine Zellwand aufweisen und die in der Lage sind, große DNA-Moleküle durch einfache chemische Transformation aufzunehmen. Wir demonstrieren die Umwandlung in die L-Form für drei verschiedene Gram-positive Arten, *Listeria monocytogenes*, *S. aureus* und *Staphylococcus xylosus*, und zeigen ihre Fähigkeit, verschiedene Phagengenome zu reaktivieren, sogar über die Gattungsgrenze hinweg.

Computergestützte Methoden sind bereits ein wesentlicher Bestandteil der modernen Wissenschaft und werden wahrscheinlich auch in Zukunft ein wesentlicher Bestandteil sein, um Fortschritte in der biologischen Forschung, insbesondere in der Gentechnik, voranzutreiben. In **Manuskript IV** haben wir eine auf maschinellem Lernen basierende Methode zur Vorhersage von Transkriptionspromotoren aus primären Phagensequenzdaten entwickelt. Wir verwendeten dieselbe biolumineszente Nutzlast aus **Manuskript**

II, um unseren Ansatz experimentell zu validieren. Wir zeigen, dass eine genaue Vorhersage der Expressionsstärke der Nutzlast auf der Grundlage der Position von Insertionsstellen hinter Promotoren mit verschiedenen vorhergesagten Expressionsstärken tatsächlich möglich ist. Dies birgt ein großes Potenzial für künftige Anwendungen zur Kontrolle der Expressionsniveaus von in das Phagenrückgrat integrierten Effektornutzlasten.

Ein Ansatz zur Steigerung der Wirksamkeit von Phagen für therapeutische Anwendungen ist die genetische Bewaffnung mit antimikrobiellen Effektor-Nutzlasten. In **Manuskript V** haben wir eine große Auswahl von Genen, welche für Nutzlasten kodieren die zuvor eine bakterizide Aktivität gegen *S. aureus* gezeigt hatten, auf ihre Eignung als Effektor-Nutzlasten in K untersucht. Obwohl wir für die meisten Nutzlastgene keine lebensfähigen Phagen erhalten konnten, gelang es uns, zwei Phagen zu entwickeln, die das Proteintoxin MazF bzw. das kurzkettige Bakteriozin Lacticin Q als genetische Nutzlast enthalten. Beide Phagen zeigten im Vergleich zu den Wildtyp-Phagen eine deutlich verbesserte *in vitro* Tötungseffizienz bei einer Auswahl von *S. aureus*-Wirten.

Mit dieser Arbeit hoffe ich, Einblicke und Fortschritte auf dem Gebiet des Phagen-Engineerings erzielt zu haben, insbesondere im Zusammenhang mit gezielten Anwendungen gegen *S. aureus*. Durch die Demonstration verschiedener Ansätze auf der Grundlage des Phagentyps und der beabsichtigten Modifikation zeigen wir, dass Phagen-Engineering ein großes Potenzial für die Entwicklung modifizierter Phagen mit verbesserten Eigenschaften für zukünftige therapeutische Anwendungen hat. Insgesamt sind Phagen-Engineering und Phagentherapie sehr vielversprechend, um die Auswirkungen der sich weltweit abzeichnenden Krise der Antibiotikaresistenz abzumildern, und haben das Potenzial, in Zukunft ein wichtiges therapeutisches Mittel zu sein.

Contents

Chapter 1

Introduction

1.1 Bacteria

Eubacteria constitute one of the three domains of life and are among the first known lifeforms to inhabit the earth. Fossils indicating the presence of simple, unicellular life date as far back as 3 billion years [1]. The earliest bacterial fossil records are those of cyanobacteria and date back 1.5 billion years [2]. Bacteria typically have a simple, unicellular structure on the micrometer scale and are ubiquitous across all ecological niches. These cover the vast majority of terrestrial [3, 4] and aquatic ecosystems [5, 6] including the Earth's crust and extreme environments such as deep-sea thermal vents and acidic hot springs [7]. Bacteria even have been shown to have direct, short-term effects on atmospheric stratification due to their significant contribution to ice nucleation and cloud formation [8–10]. Recent advances in the field of genomics have shown bacteria to have vast genomic diversity even between closely related species, in part due to a combination of genetic drift and large population sizes [11] and driven by a high frequency of recombination via homologous recombination or horizontal gene transfer [12]. The high rate of genetic drift and relatively low morphological complexity allows broad sampling of the genomic fitness landscape across all bacterial species. Although this aspect is inherently difficult to quantify, it is nevertheless evident that high levels of horizontal gene transfer and accumulation of beneficial mutations is a main contributing factor to the immense abundance and genomic diversity of bacteria and allows for rapid adaptation to changes in the environment [13–16]. Although many bacterial populations proliferate

independently in the environment, a large fraction depends on colonization of other organisms, typically eukaryotes [17]. Many symbiotic relationships with bacteria are known, such as gut microbial communities, symbiotic communities in root nodules of plants and complex bacterial-fungal interactions [18–20]. Pathogenic bacteria parasitize their host organism and niches stretch across the major domains of the tree of life, with bacterial species infecting the vast majority of plants [21] and animals [22] and thus constituting a major contributor of disease for these organisms.

1.2 The antimicrobial resistance (AMR) crisis

Prior to the discovery of antibiotics, bacterial infections were one of the leading causes of death in humans, with even small, superficial wounds bearing the potential to become infected and lead to death. Observations of the antimicrobial effects of fungal spores, particularly those of the genus penicillium, date back long before the discovery of Penicillin which is attributed to Alexander Fleming in 1928 [23]. Nevertheless, this discovery and subsequent research led to widespread availability of the first antibiotics with widespread distribution to British troops during World War II demonstrating its remarkable efficacy to treat bacterial infections [24]. The "golden age of antibiotics" proved highly effective in combating infectious diseases on a global scale with a dramatic decrease in morbidity and mortality associated with bacterial infections [25]. The effectiveness of this new class of drugs also led to a virtual stagnation into research of alternative treatment options, such as serum or phage therapy. [26, 27]. However, the first instances of antibiotic resistance began to emerge within 10 years of the widespread introduction of Penicillin [28]. The continued widespread abundance and usage of a wide variety of antibiotics led to a steady increase in the number of antibiotic resistant bacteria, with an alarming rate of newly emerging antimicrobial resistant pathogens being reported in recent decades. The vast quantities of antibiotic consumption in medicine and agriculture, coupled with the

ease of access to over-the-counter antibiotics, particularly in developing countries, have continued to fuel the emergence of new multidrug-resistant bacteria (MDR) [29]. This is paralleled by a steady decline in the amount of newly developed antibiotics [30–32]. Infectious disease remains a leading cause of death both in western, but particularly in developing countries [33, 34], where the high prevalence of e.g. MDR *Mycobacterium tuberculosis* and *Staphylococcus aureus* strains is of great concern [35–37]. In contrast, western nations typically have much easier access to antibiotics as well as better documentation and tracking of outbreaks of MDR pathogens and resulting hospitalizations [38]. Nevertheless, these countries are experiencing a troubling rate of increase of antibiotic resistance pathogens, with the World Health Organisation (WHO) speculating antimicrobial resistance to be the leading cause of death by 2050 [39, 40]. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.* (the so-called ESKAPE pathogens) are of particular concern due to their various virulence and resistance factors as well as high resistance to antibiotics in particular [41]. *Escherichia coli* is frequently included into this list (ESKAPEE pathogens) and *Salmonella spp* has also been named as a pathogen of particular concern [41, 42]. Particularly carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, carbapenem-resistant and third-generation cephalosporin-resistant *Enterobacteriaceae*, vancomycin-resistant *Enterococcus faecium* and methicillin resistant *Staphylococcus aureus* but also pathogens from community acquired infections such as clarithromycin-resistant *Helicobacter pylori*, and fluoroquinolone-resistant *Campylobacter spp.*, *Neisseria gonorrhoeae*, and *Salmonella typhi* show a dramatic increase in prevalence and there is an urgent need for the development of novel compounds to combat AMR [41, 43]. Numerous health agencies, including The European Centre for Disease Prevention and Control [44], Public Health Agency of Canada [45], US Center for Disease Control [46] and the World Health Organization [47] have published reports including this group of pathogens as a significant threat to public health and pushed towards prioritizing re-

search and development of new drugs directed against them. Overall, there is a looming threat to the golden age of antibiotics with a large range of diverse antibiotic resistance mechanisms against nearly all available antibiotics, including those used as a last resort in infection treatment [29, 48]. A renewed global and collaborative effort is necessary to combat the vast array of pathogenic bacterial strains posing a threat to continued human prosperity, with particular focus on the multitude of virulence and resistance factors which make these "super-bugs" a significant challenge to research and medicine.

1.3 Alternative antimicrobials

As mentioned previously, there is a steady decrease in the amount of newly discovered antibiotics which stands in stark contrast to the rapid emergence of new resistant strains [49]. Reports collecting data from the United States Food and Drug Administration (FDA) show a dramatic decline in newly approved antimicrobial products, marking a 56% decline in approved drugs in the last 20 years with only 3% of the approved drugs between 1998 and 2002 pertaining to antimicrobial drugs and only very few additions over the following years [31]. This problem is in part compounded by the reluctance of pharmaceutical companies to research and develop novel antimicrobials due to lack of profitability compared to e.g. chronically-administered medications [25, 50]. Conversely, just like the original isolation of Penicillin from fungi, there is hypothesized to be a plethora of bioactive natural products which could potentially be isolated from other microbial sources such as anaerobic and pathogenic bacteria, especially from bacterial populations competing for specific ecological niches, such as bacterial soil communities [51, 52]. Next to the isolation and application of natural products, further strategies are being taken into consideration for the development of novel antimicrobials. Firstly, mechanisms furthering the resistance development of bacteria to natural products can be targeted, for instance by altering the original chemical structure of naturally occurring

agents [53, 54] or by using compounds which directly inhibit the bacterial mechanisms involved in resistance development, such as β-lactamase inhibitors to restore antibiotic efficacy against β-lactame resistant strains. [55–58]. Another approach is the discovery of novel antimicrobial compounds, particularly ones which differ in structure or mechanism of action (MOA) compared to conventional antibiotics [59–61], inhibition of bacterial virulence factors [62], antimicrobial nanoparticles [63] antimicrobial peptides and peptidomimetics [64–67], bacteriocins [68, 69], enzybiotics (lysins/endolysins) [70, 71], antisense oligonucleotides [72, 73] CRISPR-Cas [74–76], and phage therapy [77–79]. The various approaches including their background, rationale and methodology will be expanded upon in the following sections. An overview of the main approaches including example compounds are given in **Table 1**.

1.3.1 Discovery of novel antimicrobial compounds

The prediction of potential metabolic compounds and their targeting of proteins and pathways essential for bacterial survival has experienced a surge in recent years, mostly stemming from sophisticated machine learning algorithms capable of predicting such compounds as well as the fast accumulation of vast amounts of bacterial genomic data [80, 81]. This *in vitro* discovery of bacterial inhibitors remains difficult, especially since the *in vivo* efficacy is not guaranteed. One limitation, for example, is the permeability of the bacterial surface for a predicted synthetic compound with inhibitory effect. Therefore, the traditional method of identifying secondary metabolites with antimicrobial activity from microorganisms present in the environment remains the method of choice. These potential compounds are hypothesized to be abundantly present in nature, ranging from antibiotic producing microorganisms in exotic niches, such as marine invertebrates and algae, to insects and invertebrate organisms, for example as symbionts of plants [82–84]. The human microbiome is a complex and diverse bacterial ecosystem and offers a second system with large potential for the isolation of novel antimicrobials. Strong

Table 1: The table lists a variety of antimicrobial compounds which present alternatives to currently used antibiotics. These are classified based on their molecular class and mode of action against bacterial pathogens. The main mechanisms, their background and prominent examples are given.

Mechanism	Background	Examples
Targeting of alternative bacterial proteins and pathways	Isolation of novel antimicrobials against new pathways and proteins essential for bacterial survival. High abundance of naturally occuring compounds as well as novel methods for discovery of compounds from metagenomic sequence data.	Lugdunin, Tetarimycin, Humimycin
Virulence blockers	Disarming of bacteria by targeting virulence factors directly. Conserves function of beneficial populations and has a low chance of resistance evolution.	Pilicides, Erianin, Savirin, Licoflavonol, Salicylidene acyl hydrazides
Nanoparticles	Penetration into bacterial cells, interference with formation and structural integrity of the cell membrane	Silver, Titanium oxide
Antimicrobial Peptides and Peptidomimetics	Membrane permeabilization, inhibition of protein, DNA and RNA synthesis	CSA-131, LL-37, Iseganan
Bacteriocins	Produced by vast majority of bacteria and archaea species. The producer strain is typically protected by simultaneously expressed immune factors.	Nisin, LFF571
Enzybiotics	Degradation of bacterial cell wall	LysK, LysAB2, Lysostaphin, SPN9CC
Antisense oligonucleotides	Modify gene expression either by blocking translation or stimulating RNase H1-based cleavage	phosphorodiamidate morpholino oligomer, peptide-LNA (PLNA), peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs)
CRISPR	Bacterial DNA damage via delivery of Cas nucleases	CRISPR-Dx, pDB121mecA

evolutionary pressure among coexisting bacteria and constant niche competition leads to the presence of potent antimicrobial peptides. One such example is lugdunin, a novel thiazolidine-containing cyclic peptide produced by the human commensal *Staphylococcus lugdunensis*. Lugdunin has been shown to have strong bactericidal activity against major human pathogens such as *S. aureus* without leading to resistance development [59]. Furthermore, integrating metagenomic sequence data from the human microbiome into novel bioinformatic approaches led to the identification of two novel, naturally occurring antimicrobials tetarimycin and humimycin, by means of the identification of biosynthetic gene clusters (BGCs) [60, 85] and prediction of antimicrobial protein structures [61], respectively.

1.3.2 Virulence blockers

An elegant alternative to conventional antimicrobials is the use of compounds with MOA directed against specific bacterial virulence factors, thereby reducing the pathogenicity of the bacterial population and allowing for rapid and effective clearing of the infection by the host immune system. The unique advantage of this approach is that it effectively "disarms" the bacteria without inducing an evolutionary fitness cost for the bacterial population on an intra-host level and thereby prevents resistance-emergence. Furthermore, commensal bacteria tend to lack most factors targeted by anti-virulence agents and the host's beneficial microbiome is largely preserved [86]. There are numerous compounds targeting a wide variety of virulence factors. Inhibition of factors governing host-cell attachment have been shown to drastically reduce the translocation and proliferation in host tissues in several cases [87, 88]. One such example is the use of bicyclic 2-pyridones to inhibit pilus biogenesis in uropathogenic *E. coli* [89]. Manipulation of host signaling pathways responsible for immune modulation can be targeted by bacterial virulence factors, as is the case for the anthrax toxin of Bacillus anthracis [90]. Targeting these compounds specifically has been shown to reduce bacterial virulence while increasing

host immune response, such as LpxC-inhibitors to block the synthesis of the lipid A endotoxin present in most Gram-negative bacteria [91–93]. Other promising approaches include small molecule inhibitors interfering with biofilm formation [94], targeting of compounds involved in pathways of global virulence regulation [95–97], interference in quorum sensing networks [98, 99], suppression of bacterial toxins [100–102], the dismantling of bacterial membrane microdomains [103, 104], targeting of bacterial secretion systems [105, 106] and engineered liposomes to sequester bacterial toxins produced during *S. aureus* and *S. pneumoniae* infection [107, 108]. Nevertheless, further infection models and larger clinical trials will be needed in the future to further assess the efficacy of these compounds [109, 110].

1.3.3 Nanoparticles

Material science on a nanoscale has undergone tremendous advancements in the last decade. The ability to precisely modulate properties unique to nanoparticles such as optical, electronic, magnetic, chemical, mechanical and catalytical [111, 112] has allowed their application in numerous fields including biomedicine, electronics, food control and environmental wastewater purification [111, 113]. Metals like zinc, silver and copper are well known to exhibit antibacterial activity and iron oxide is also associated with decreased viability of bacterial populations [114]. The general MOA are shared across most classes of nanoparticles and consist of attraction to bacterial membranes through electrostatic interactions, penetration into bacterial cell bodies, interference with cell membranes and the generation of reactive oxygen species [115, 116]. Although nanoparticles have a significantly reduced effect on eukaryotic cells compared to bacteria, nanoparticle concentrations needed to effectively inhibit bacterial activity have been shown to have local cytotoxic effects and ideally methods confining the particles to the site of the infection should be used [117, 118].

1.3.4 Antimicrobial peptides and peptidomimetics

Antimicrobial peptides (AMPs, host defense peptides (HDPs)) are ubiquitous across all domains of life and make up part of the host's innate immune response. Bacterial AMPs are expressed by the producer strain in order to gain a competitive advantage during niche-competition which is achieved through peptide-mediated killing of surrounding bacteria [119]. They are typically short (7-100 amino acids (aa)) with the activity being based on their secondary structure and charge distribution. AMPs are usually present in either linear or cyclic structure, either with amphipathic α-helices or disulfide bridge-containing β-sheets [120, 121]. AMPs are historically known to have a broad MOA by manipulating membrane permeability. Advances in transcriptomics, proteomics and lipidomics, as well as computational tools to model molecular dynamics, have allowed for the elucidation of novel intracellular MOA, such as inhibition of protein, DNA and RNA synthesis [122, 123]. Unfortunately, AMPs typically have a low plasma half-life, show unexpected *in vivo* toxicity and tend to have a higher MIC than conventional antibiotics, although this is not always the case [124]. These factors frequently lead to failure of AMPs in preclinical studies [125–127]. Due to the broad MOA targeting the bacterial membrane, development of resistance to AMPs is believed to be unlikely. Nevertheless, studies have shown cases where resistance to AMPs arose after prolonged exposure [128]. The use of synthetic peptides mimicking the structural conformation of naturally occurring AMPs (peptidomimetics) is an important discovery and promising alternative. An important factor is the use of non-canonical amino acids or non-peptidic scaffolds to mitigate the aforementioned pharmacokinetic drawbacks of naturally occurring peptides [129]. A number of studies show promising results in the application of peptidomimetics against a variety of Gram-positive and -negative bacterial pathogens [130–133]. Although resistance evolution is expected to be exceedingly rare compared to naturally occuring AMPs, cross-resistance development of *S. aureus* under exposure to the synthetic peptide pexiganan has been reported [134].

1.3.5 Bacteriocins

One important subclass of antimicrobial peptides and proteins are bacteriocins. Bacteriocins are thought to be produced by the vast majority of bacteria and archaea and thus are widely abundant in the environment [135]. Bacteriocins are expressed and secreted by a producer strain to compete against other other bacterial species present in the surrounding environment. The producer strain simultaneously synthesizes specific immunity proteins to protect against toxicity of the expressed bacteriocin [136]. Bacteriocins have a diverse set of functions, which can be broadly separated into mechanisms which have an effect on the cell envelope or act intracellularly [68], as well as those which undergo post-translational modifications (class I bacteriocins) and those which remain unaltered (class II bacteriocins) [68]. They further exhibit a wide range of specificity, with some bacteriocins acting against closely-related species as while others show activity only against non-related species [69]. This diverse set of specificity and mechanisms, as well as a high tolerance to abiotic factors such as heat and UV-radiation, make bacteriocins a promising alternative to antibiotics [137]. Colicin-like bacteriocins (CLBs) are some of the best characterized bacteriocins, are produced by Gram-negative bacteria and have shown high in *vivo potency*, low immunogenicity and low levels of resistance [138, 139]. Another class of Gram-negative-produced bacteriocins are tailocins, which resemble the tail structure of bacteriophages and have been specifically adapted by bacteria to incur lethal membrane damage to target cells [140]. Bacteriocins and engineered variants thereof have been used successfully in sectors such as food industry and medicine [68, 141]. For example, Nisin is a class I bacteriocin produced by *Lactococcus lactis*, has been reported to inhibit the growth of Gram-positive bacteria, including *Clostridium* and *Bacillus* spores, and has found a wide range of applications in the meat industry [142]. A further class I bacteriocin, *Planobispora rosea*-produced GE2270 A, shows potent activity against *Clostridium difficile* and other Gram-positive bacteria [143]. This compound has further been developed into a synthetic derivative LFF571 (Novartis) which shows has

shown great potential for clinical application in treating infections caused by a variety Gram-positive pathogens [144].

1.3.6 Enzybiotics (lysins and endolysins)

Lysins and endolysins are cell-wall active enzymes typically encoded by bacteriophages to aid them during the infection of bacterial cells. Lysins, often called virion-associated lysins (VALs), act during cell attachment and facilitate local degradation of the bacterial cell wall, therefore allowing for penetration of the phage tail tube and subsequent injection of viral DNA [145, 146]. At the end of the infection cycle, endolysins cross the cytoplasmic membrane through pores formed by the phage encoded holin protein and subsequently degrade the peptidoglycan polymers leading to osmotic cell lysis and release of phage progeny [147, 148]. Like the phages that encode them, endolysins typically have a very high specificity on a species or even a serovar level, thereby posing a directed treatment option with little or no effect on the commensal microbiome [149]. One key feature of endolysins is the modular domain architecture, particularly in Gram-positive targeting endolysins, which can be exploited to engineer proteins with enhanced or specifically tailored bactericidal activtiy. The protein contains distinct enzymatic and cell-wall binding domains (CBDs) which can be easily engineered, for example, by domain truncation or deletions [150], direct mutagenesis [151–153] and domain swapping methods [154–158]. Gram-negative endolysins are globular in structure with the CBD typically absent. The protective outer membrane (OM) of Gram-negative cells prevent the external action of lysins. Examples of Gram-negative endolysins with the ability to permeate the OM are sparse [159, 160] making the translocation of lysin proteins across the outer membrane a crucial component for the engineering of Gram-negative targeting endolysins [161]. Different engineering strategies have been attempted in this context, including the fusion with OM-penetrating peptides or translocation/receptor binding domains [162–164]. Another approach is the encapsulation of Gram-negative endolysins

into e.g. cationic liposomes or chitosan nanoparticles [165, 166].

1.3.7 Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are small, single-stranded nucleic acids which are complementary to their target mRNA sequence and modify gene expression, either by blocking translation or stimulating RNase H1-based cleavage [167]. By rationale selection of candidate genes and design of corresponding ASOs, critical functions and pathways essential for bacterial growth, such as fatty acid biosynthesis, cell division and RNA polymerase activity, can be targeted specifically [168, 169]. ASOs can be grouped based on structure and function. Phosphorothioates are modified in the form of a sulfur atom instead of one of the non-bridging oxygen atoms on the phosphate linkage which renders them resistant to nuclease activity [170, 171]. Locked nucleic acids (LNA) are modified RNA bases that consist of 2'-O,4'-C-methylene bicyclonucleoside monomers and are capable of recognizing and binding single stranded DNA and RNA [172]. Like phosphorthiotes, LNAs are stable to nuclease activity and have a high DNA/RNA binding affinity and additionally exhibit low toxicity [173]. Studies testing the effect of various locked nucleic acids on bacteria have been conducted in the past and show potential against pathogens such as *E. faecalis*, *S. aureus* [174, 175] and *Brucella suis* [176].

1.3.8 CRISPR-Cas

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nucleases (Cas) are an essential component of the prokaryotic adaptive immune system and recognize and defend against exogenous viral or plasmid DNA. These RNA-guided nucleases are efficient in cleaving genomic DNA, exhibit high target specificity and have been adapted into one of the most widely used genome engineering technologies in molecular biology to date [177, 178]. Numerous studies have demonstrated that CRISPR systems are associated with higher bacterial antibiotic susceptibility. Further-

more, CRISPR systems have been engineered to reliably detect and control for infections displaying the emergence of antimicrobial resistance [179–182]. Furthermore, CRISPR can be used to target antibiotic resistance genes (ARGs) with high sensitivity and specificity [182]. Plasmids, extracellular vesicles, phages, and nanoparticle vectors can be used as vectors to deliver CRISPR-Cas systems into bacterial cells and reduce the level of bacterial drug resistance by targeting drug-resistant genes and plasmids [183]. CRISPR, therefore, has large implications on mitigating the spread of antibiotic resistance genes among bacterial populations, particularly in combination with antibiotic treatment regiments, which are exceedingly prone to resistance development.

1.3.9 Phage therapy

Another natural occurring antimicrobial heralded as a promising alternative to antibiotics is that of bacterial viruses (bacteriophages or phages). The potential for phages as a therapeutic will be covered in greater detail in the following section. Briefly, the vast diversity, abundance and high specificity of phages allows for targeted killing of bacterial pathogens. The high specificity results in very few off-target effects, such as harming of beneficial microbiota. Harmful effects on eukaryotic cells are also not known. Furthermore, the relative simplicity of the phage machinery combined with advances in genetic engineering and synthetic biology have allowed the development of modified phages with enhanced specificity, lytic ability and immunogenicity.

1.4 Bacteriophages

Bacteria infecting viruses, termed bacteriophages or simply phages, are the most abundant biological entity on earth with the number of phage particles estimated to be in the order of 10^{31} [184, 185]. They show an incredible diversity, with widely accepted estimates ranging up to to 100 million individual phage species [186]. Although the precise

evolutionary origin of phages is difficult to define, they are arguably an ancient group and speculated to be at the level of the last universal common ancestors of eubacteria, archaea and eukarya [187]. Phages infect roughly 10^{24} bacteria every second and, like their bacterial hosts, are present throughout all ecological niches on earth, including deep sea hydrothermal vents and dispersion across the upper stratosphere [188–190]. The steady stream of vast amounts of genomic and metagenomic data means constant revisions to the taxonomic classification of bacteriophages, which is maintained by the International Committee on Taxonomy of Viruses (ICTV) [191]. Phages come with a wide range of morphologies, which is also reflected in the diversity of genome sizes, ranging from 4kb to 600kb [192]. The pangenome of phages is immense and genomic analyses continue to outline its diversity and complexity, which is in part fueled by the rapid co-evolution of bacteria and phages and the corresponding high rates of mutation and horizontal gene transfer [193, 194]. The vast majority of known phages are tailed and belong to the order Caudovirales. They can further be split into three classes– Myo-, Sipho- and Podoviridae –on the basis of their tail morphology, although the evolutionary basis of this classification is debated and phage taxonomy is shifting fast from morphologicalal characteristics to genome-based characterization [195]. Most major bacterial pathogens have been shown to have phages of the order Caudovirales capable of infecting clinically relevant strains, particularly those with high levels of resistance evolution, making these phages of great importance for phage therapy in general [196]. The majority of phages can be classified as being virulent or temperate and follow a strictly lytic or a lysogenic life cycle, respectively [197]. Phage infection begins with the attachment to the surface of the host bacterium using highly specific receptor binding proteins. Attachment is typically followed by localized permeabilization of the cell wall using lytic proteins present on the phage attachment apparatus and injection of the phage DNA into the bacterial cytoplasm [198, 199]. For virulent phages, the injected genomic phage DNA is circularized, and the host's cellular machinery begins with replication of the phage genome followed

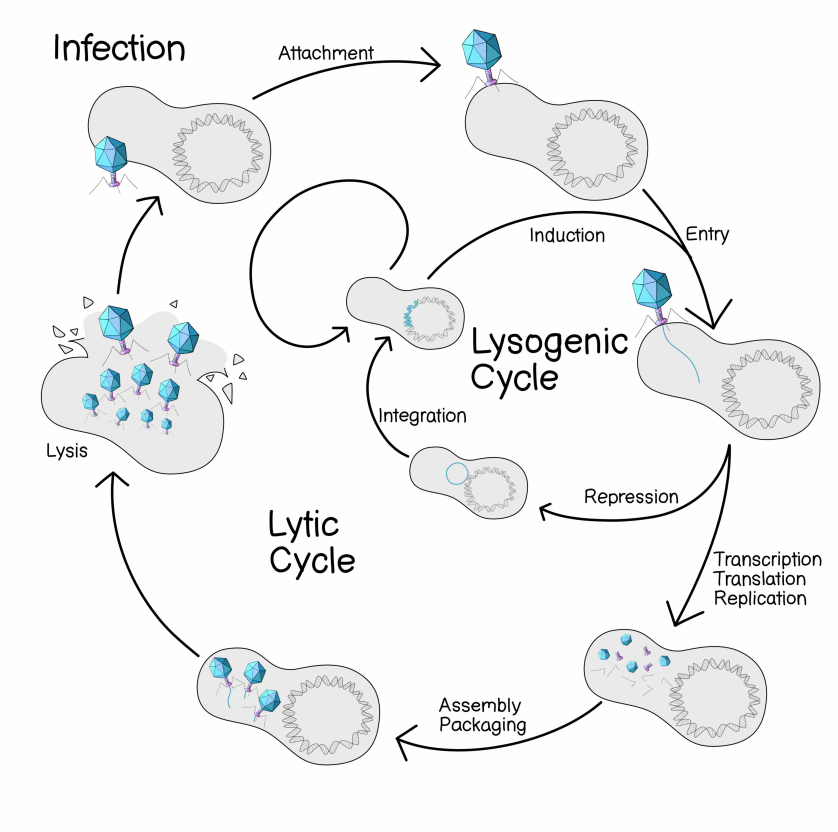

Figure 1: Lytic and lysogenic life cycles of phage infection. The figure is loosely adapted from [202].

by expression and assembly of phage structural proteins. The assembled phage progeny is then released from the cell, usually by disruption of the cell envelope and sudden lysis, exposing the newly produced infectious phage particles and allowing for repetition of the infection cycle on surrounding host bacteria [200]. Depending on numerous factors such as environmental conditions, genetic compatibility with the host, host physiological state and density, temperate phages can enter into non-virulent infection, also known as lysogeny, where the phage DNA is integrated into the host cell genome and production of phage progeny and cell lysis does not occur. [201]. An overview of the phage lytic and lysogenic life cycles is depicted in **Figure 1**. The molecular pathways and abiotic

factors involved in the control of this process stand in a complex and intricate relationship and have been extensively reviewed in the past [203–206]. Generally, there are clear parallels between the phage lysis-lysogeny decision and programmed cell death/apoptosis ubiquitous to all cellular life forms [207]. During lysogeny, the phage DNA is integrated into the bacterial chromosome to become a prophage, a state which persists in a latent or dormant state without host cell lysis or production of phage progeny [208]. Although stable integration into the bacterial chromosome occurs in the majority of temperate phages, it is not stringently necessary, as some phages have been observed to persist as low copy number plasmids [209, 210]. This allows the phage to replicate along with the bacterial DNA, a state which is maintained through the active repression of phage lytic genes. Furthermore, this repression also prevents the subsequent infection of phages which encode the same or similar repressors, effectively mediating a form of immunity for the host cell and often termed "superinfection exclusion" [211]. The process can be reversed by a variety of abiotic stress factors, including DNA damage by ultraviolet (UV) radiation [212], presence of nutrients [213, 214] and salinity [215]. Biotic factors, such as the communication of phages with their progeny to regulate the lysis-lysogeny decision as seen in the case of the arbitrium system employed by phages of the SPbeta family, are also known [216]. The switch back to lytic production is initiated by the excision of the prophage genome and followed by the expression of lytic genes promoting DNA replication, the assembly of progeny phage particles and lysis of the host cell bacterium. Spontaneous prophage induction is also thought to be possible and has been observed in the absence of any obvious stress factors [217].

The resistance to phage infection following prophage integration and repression of lytic proteins has problematic implications for the application of phages in a clinical context and reduces the selection of phages viable for therapeutic purposes down to those known to have strictly lytic life cycles. Furthermore, temperate phages have shown elevated levels of generalized transduction and horizontal transfer of antibiotic-resistance

genes [218–220] although the process is also known to occur in strictly lytic phages [221]. A third life cycle exists, typically associated with filamentous phages. Here, rather than killing the bacterial cell through production of lytic proteins, filamentous phages produce pore-forming proteins which assemble at the bacterial membrane and allow for the extracellular secretion of assembled virus particles [222, 223].

1.5 Phage therapy as an alternative to antibiotics

Phages were discovered independently in 1915 by the British pathologist Frederick Twort [224] and in 1917 by the French-Canadian microbiologist Félix d'Hérelle [225]. Although the therapeutic potential of phages was recognized early in the 20th century, lacking knowledge of the underlying molecular mechanisms of phages coupled with the advent of the antibiotic era led to research into phage therapy slipping largely out of focus, particularly in western countries [226]. A variety of phage products were continuously developed and applied throughout the former Soviet Union and are still in use today – one of the most renowned examples being the Eliava Institute of Bacteriophages, Microbiology and Virology in Tblisi, Georgia [227]. A combination of cold-war era politics, poorly documented research and non-randomized clinical trials conducted in the former Soviet Union led to bacteriophage research being cast in a bad light and widely criticized by western countries [227–229]. The onset of the global antimicrobial resistance crisis, however, has led to rekindled interest and a surge in the amount of research being conducted on phages [230, 231]. In addition to increasing the public awareness and clinical guidelines for correct application of antibiotics [232], the re-evaluation of the potential for widespread implementation of bacteriophage therapy is proceeding at an accelerated pace. Especially the publication of several recent reports describing exceptionally positive patient outcomes after the application of experimental phage therapy has spurred on the revival of phage applications in the Western world [233–235]. Phage products such as

Intestiphage or Pyophage, which are produced by the Eliava Institute in Georgia, have been marketed openly and free of prescription for decades [236]. In recent years, several European companies have followed suit. Although effective, controlled and constant production of commercialized phage products has yet to be achieved, companies such as Omnilytics Inc. (Sandy, UT, United States) and Intralytix Inc. (Baltimore, MD, United States) as well as the European companies Micreos BV (Wageningen, Netherlands) and Fink Tec (Hamm, Germany), have established, and are actively distributing, a variety of phage products, such as disinfectants against *Salmonella*, *Escherichia coli* and *Listeria monocytogenes*. These products mostly find application in the food and agricultural industry. However, there are already some bacteriophage-derived healthcare products on the market targeted against human pathogens e.g, *S. aureus* [237]. Also promising for the future of phage therapeutics is the launch of a phage translational research center at the University of California, San Diego (UCSD). Funded by a $1.2 million research grant, the new Center for Innovative Phage Applications and Therapeutics (IPATH) aims at "applying the same principles of clinical evaluation and development to phage therapy that would be applied to any other therapeutic entity" [238]. Also promising is the major player of Big Pharma Johnson & Johnson investing over a billion US dollars into Locus Biosciences to develop CRISPR phages and the Israeli company BiomX, which is looking to apply phage therapy to dysbiosis of the microbiome [238].

The number of clinical or safety trials as well as compassionate use cases is also on a steady rise. Between 2005 and 2021, 13 clinical or safety trials were conducted across numerous countries against a variety of pathogens, clinical conditions, and modes of administration [239–251]. To date, phage therapy is typically used as a last resort when all other treatment options have failed. A large and growing number of compassionate use cases revealed promising perspectives with their positive clinical results (40%-100% in case studies with more than one participant). Only very few cases showed the pathogen of interest developing resistance to the phage over the course of the treatment [252–275].

The success in treating bacterial infection using phages as an alternative to antibiotics is evident from these case studies. However, the total amount of clinical trials remains relatively low compared to other medical fields and further critical evaluation of factors such as resistance development, immunogenicity and pharmacokinetics should be conducted [276, 277]. Furthermore, advances in high-throughput screening for phages capable of infecting a specific bacterial strain as well as upscaling of physical and digital phage banks have the potential to greatly increase the efficiency and distribution scale of phage therapy as a whole.

1.5.1 Advantages of phages over antibiotics to treat bacterial infections

There are numerous benefits when it comes to using phages to treat bacterial infections when compared to conventional antibiotic treatment regimens. Phages have very high specificity for their hosts. Most are believed to only be capable of infecting a narrow range of closely related bacteria at the species or even serovar level [278]. This feature is one key advantage over antibiotics. The correct application of phage therapy can specifically target the infection while retaining the integrity of the human commensal microbiome. The disruption of the commensal microbiome is an unwanted side effect of antibiotic treatment, specifically for broad spectrum antibiotics, where significant side effects can occur due to the depletion of beneficial bacterial communities from the body [279–281]. Contrary to antibiotics, treatment with phages has not shown any adverse side effects to patients and immunogenicity remains relatively low [282]. Although resistance of bacteria against the phages can occur, phages are a fast co-evolving system and can potentially overcome this resistance through adaptations of their own during the course of the treatment [283]. One fascinating example is presented by phages encoding diversity-generating retroelements, which allow for rapid adaptation to bacterial surface receptor modifications as well as host tropism switching [284]. Furthermore, the application of phage cocktails containing several phages targeted against the same bacterium makes

resistance development against the entire phage population exceedingly unlikely, provided the cocktail is designed such that the contained phages have different receptors and/or infection mechanisms [285].

The ubiquity and abundance of phages in nature present a virtually endless and easily accessible source of new phage variants, which are quick and easy to isolate. Phage resistance comes at a cost to the bacterium and has been associated with reduced virulence [286]. Finally, there also appears to be an inherent trade off in fitness cost for bacteria between evolving phage and antibiotic resistance and synergistic effects of co-treatment with phage-antibiotic combinations have been observed in the past. [287, 288].

1.5.2 Disadvantages of phages over antibiotics to treat bacterial infections

Several caveats are associated with the use of phages as antimicrobials. Temperate phages are not suited for phage therapy due to their ability to integrate into the bacterial genome and render the cell phage resistant via mechanisms such as superinfection exclusion [289]. Phages intended for use in therapy must be well characterized. The full genome sequence is necessary to exclude the presence of potentially toxic genes [290–292]. The efficiency of infection against their target bacterium should be tested, as factors such as poor adsorption properties, low potential to evade bacterial defenses and poor replication characteristics can significantly hamper treatment efficacy [293]. Furthermore, as described previously, the onset of the development of bacterial resistance mechanisms against phage infection can be rapid. Numerous mechanisms such as blocking of phage adsorption through receptor mutagenesis or acquisition of adaptive immune systems such as restriction-modification or CRISPR have been identified in the past [294–296]. A further concern is the potential spread of antibiotic resistance genes by means of generalized and specialized transduction, processes which are associated with some phages [297].

Pharmacokinetic information is required to determine adequate dosage and the most

effective treatment parameters [291]. Specifically, effects of phage immunogenicity can lead to the rapid depletion of phages in the bloodstream and liver, even in the absence of phage-specific antibodies [298–300]. Conversely, phage-mediated bacterial lysis can lead to the release of endotoxins such as lipopolysaccharides (LPS), which results in high levels of inflammation and can be dangerous to the patient [301, 302]. To date, most cases of phage therapy are against persistent or chronic infections, where antibiotic treatment has failed. This stands in contrast to observations that phage therapy tends to be more effective in fully clearing the infection when administered early, before the formation of complex, heterogeneous biofilms [303]. The immediate environment of phage-bacteria interaction also has direct implications on the effectiveness of phage therapy. Complex matrices such as bovine raw milk and human whole blood, which are relevant for clinical and veterinary applications, respectively, can have a significant impact on phage infectivity. E.g., the binding of Whey proteins, which are present in bovine milk, to bacteriophage receptors on the bacterial cell surface competitively inhibit phage adsorption [304]. Environmental and serotype-specific inhibitory effects on phage infection have also been observed in laboratory cheese models involving *Listeria monocytogenes* and have strong implications for the usage of phages in the dairy industry [305]. Correct storage and handling of phages must also be taken into account. Preservation of phages over prolonged periods is usually possible, however, individual phages can exhibit exceeding instability with rapid deterioration of their titer over time. This instability is observed across all manner of phage types and does not appear to correlate with host strain or phage morphology, albeit for non-tailed phages, which in general appear much less stable than their tailed counterparts [306]. This also has strong implications for the production of phages for clinical applications, which can be significantly hampered due to instability of specific phages during certain production processes [307]. These factors must be taken into consideration for formulation development of phage therapy products to ensure that, for example, phage titers are not significantly reduced during handling prior to patient

treatment.

1.5.3 Bacterial defense strategies

Next to phage-specific and environmental characteristics inhibiting phage infectivity, bacteria have evolved a plethora of defense strategies. These present a dynamic set of adaptations which are under constant selective pressure and co-evolving in the presence of phage predation. These mechanisms can be broadly split into two groups. Effects displayed extracellularly or on the bacterial surface typically serve to inhibit phage adsorption or genome delivery. On the other hand, intracellular MOA forms a type of immune system which, analogous to mammalian immunity, has both innate and adaptive components. Different bacterial phage-resistance mechanisms, their MOA, as well as their analogous components in animal immunity are given in **Table 2**.

One of the main factors determining the host range of a particular phage are receptor molecules on the bacterial surface and the corresponding phage-encoded receptor binding proteins, which allows for species-specific attachment of the phage to the bacterial cell prior to DNA delivery [289]. Consequently, bacteria have evolved a number of strategies to block adsorption of phage virions.

1.5.4 Preventing phage adsorption

By masking or mutating surface receptors, bacteria can alter their exposed residues or induce structural confirmation changes [294, 295, 309]. The production of extracellular matrices can physically mask the surface receptors from phage association [310–312]. By exploiting competitive receptor inhibitors bacteria can drastically reduce available attachment sites and thereby evade phage predation [313]. Some bacterial populations have been shown to enter a mode of differential surface receptor expression. In a mechanism termed phase variation, a binary switch between expression and degradation of receptor proteins allows a subpopulation of the bacteria to remain resistant to phage

Table 2: Bacterial 'immunity' against bacteriophages. From [308]

Mechanism	Description	Immune system analog
Encounter blocks	Extracellular polymeric substances blocking virion approach to bacterial surfaces, e.g., capsules	Anatomical or physiological barriers, e.g., keratinized skin, mucous, etc.
Adsorption resistance (envelope-level resistance)	Absence of necessary receptor molecules on bacterial surfaces, resulting in binding failure	Racial or species immunity
Immunity to superinfection (homoimmunity)	Blocks on phage movement while in association with host, in this case preventing entrance into host cytoplasm during adsorption	Barrier responses to wounding (e.g., clotting); localization of inflammatory responses
Immunity to superinfection (homoimmunity)	Recognition of specific phage-associated motifs resulting in blocks on phage replication	Lectin and alternative complement pathways; response to recognition by toll-like receptors
Abortive infection	Killing of phages but at cost of death of individual, phage-exposed bacteria	Apoptosis induced via cell-mediated immunity; action of interferon
Restriction-modification	Generic features of organisms are targeted (recognition sequences found in DNA); equivalent host features are protected	Complement, especially alternative pathway; recognition by natural killer cells of absence of class I MHC; recognition of absence of CpG motif methylation
Phage growth limitation system	Tagging of phages for elimination by clonally related cells	Opsonization
CRISPR	Phage resistance via acquisition of novel-to-host DNA sequence	Adaptive immunity

adsorption [314].

1.5.5 Preventing phage DNA entry

Superinfection exclusion (Sie) (see section *Bacteriophages*) is mediated by proteins which associate with the cell membrane and prevent DNA delivery of specific phages. These are often encoded by prophages to protect their host cell from super infection [313]. A number of different Sie systems have been previously identified in both Gram-negative and positive species although the exact processes preventing superinfection for many phage/host pairs remains to be elucidated [315–317].

1.5.6 Degradation of foreign DNA

Restriction-modification (RM), clustered regularly interspaced short palindromic repeats (CRISPR) systems and CRISPR-associated (Cas) proteins are components of the bacterial immune system which lead to the degradation of foreign DNA. These processes differ in their mechanisms and are responsible for inducing the bacterial innate and adaptive immune reactions, respectively [313]. Restriction-modification systems typically consist of two or more genes encoding a restriction endonuclease and DNA methyltransferase (MTase), which are responsible for cleaving and methylating the DNA, respectively. Site-specific recognition allows for targeted restriction of exogenous DNA by the restriction enzyme, while cut sites on the bacterial genome are protected by complementary site-specific methylation [318]. RM systems are ubiquitous among bacteria and can display significant diversity even among the same species by replicating via mobile genetic elements rather than integrating into the bacterial chromosome [319]. Conversely, phages can evade restriction by RM systems through mutating endonuclease recognition sites on the phage genome [314]. Restriction of the *E. coli* encoded endonuclease EcoRII requires two cut sites in close proximity. Phages T3 and T7 appear to have evolved in a manner so that all potential EcoRII cut sites are spaced far enough apart to avoid EcoRII

activity [320]. Phage T7 also evolved resistance to a further RM system, EcoP1I, which requires two inversely oriented recognition sites, whereas all recognition sites on the T7 genome are in the same orientation [321]. A further strategy undermining bacterial RM activity is presented in the form of incorporating unusual bases into the phage genome, which are not recognized by bacterial encoded enzymes [322]. Transient occlusion of restriction sites on the phage genome by means of accessory proteins, which bind to the phage DNA and prevent recognition of endogenous RM systems, has also been observed [323]. Phages can also take direct action on bacterial RM. This can occur via enzymes stimulating host-encoded MTases to modify phage DNA to protect it from restriction. Another mechanisms is the degradation of cofactors associated with endonuclease function [324]. Phages encoding their own MTases to protect their genome from restriction are also known [325, 326].

CRISPR provides bacteria a form of adaptive immunity against phages and other mobile genetic elements and forms a genetic memory of past infections. By incorporating short sequences of foreign DNA into the CRISPR array, these are subsequently transcribed into short interfering RNAs (crRNAs) which in turn serve as target recognition sequence of a ribonucleoprotein for sequence-specific induction of DNA double strand breaks [327]. Although this process is highly efficient in providing adapted long-term immunity against recurring infection, phages have shown rapid adaptation to acquired CRISPR immunity, the simplest of which are single nucleotide mutations and deletions to alter the CRISPR recognition site on the phage genome [328]. This in turn can be countered by a bacterial-encoded mechanism of elevated rates of spacer acquisitions during an active infection, allowing for higher variation among the phage-specific target sequences and thereby reducing the chance of escape mutations [329, 330]. The presence of anti-CRISPR [331] and anti-anti-CRISPR [332] genes on the phage and bacterial chromosome, respectively, further demonstrates the vast complexity and dynamic nature of phage-bacterium co-evolution.

1.5.7 Abortive infection and toxin-antitoxin systems

Programmed cell-death (PCD) is a form of "altruistic suicide" ubiquitous among both prokaryotic and eukaryotic cells. In response to viral infection, cellular self-destruct mechanisms are triggered to kill the cell before the infection can progress [333]. PCD in bacteria usually occurs in response to phages and is mediated by a number of mechanisms also termed phage exclusion systems or abortive infection [314]. Abortive infection leads either to cell-death or metabolic arrest with the prime goal of preventing the release of phage progeny and the spread of the infection to surrounding cells [334, 335]. A variety of abortive infection (Abi) systems displaying diverse sets of mechanisms have been studied, starting with the first discovered Abi in the form of the repressed lambda prophage encoded Rex system [336, 337]. Further mechanisms include abolishing DNA replication and late gene transcription [338], inhibition of the host translation machinery [339, 340], or phosphorylation of large numbers of proteins involved in critical cellular functions [341]. An additional line of defense against phage infection is mediated by toxin-antitoxin (TA) systems, although their role in Abi as a phage defense mechanism remains under dispute [335, 342]. TA systems are typically made up of a co-transcribed pair of genes with opposing functionality; the first being toxic and the second providing immunity against the toxin [343]. TA systems can be divided into multiple types depending on their mode of action, the most common being type II TA systems where the antitoxin physically binds and inhibits the toxin and is downregulated in response to cellular stress signals [344].

1.6 Phage engineering for enhancement of therapeutic potential

As mentioned previously, although phages show a promising alternative to antibiotics, several caveats are associated with their use as antimicrobial therapeutics. Especially

when applying naturally occurring phage variants, these must be well characterized to verify the absence of potentially toxic genes as well as adherence to a strictly lytic life cycle to avoid prophage integration. Furthermore, the phage in question should be able to efficiently lyse the target bacteria, have a wide range of infectivity across strains within a particular pathogenic species, low chance of bacterial phage-resistance development and no undesired immune reactions when administered in humans – traits which are rarely all exhibited by a single, naturally occurring phage [345]. One approach to overcoming these barriers is the use of genetic engineering to create new phage variants with enhanced function. This can be achieved by elevating the bactericidal efficacy or reducing the chance of resistance evolution by the bacterium. Modification of surface receptor proteins is one common escape strategy employed by bacteria to evade phage predation. By engineering phage-encoded receptor binding proteins, either by directed evolution [346, 347] or targeted modifications [348], the host range of phages could be expanded to previously non-compatible hosts. In the case of directed evolution experiments, the resistance evolution of the bacterial population could additionally be decreased through the abundance of functionally diverse receptor binding proteins in the phage population [347]. Another approach to overcoming bacterial resistance evolution is using phage encoded anti-CRISPR proteins to provide the phage with tools to evade bacterial intracellular defense after genome delivery into the cell [349]. Another approach used phages who are naively sensitive to bacterial defense mechanisms and engineered them to contain a functional methyltransferase, therefore generating resistance against endogenous RM systems [350].

Another viable approach is the introduction of antimicrobial payloads into the phage genome, which are co-expressed during phage infection and can be selected to have intracellular or extracellular effects, depending on the intended strategy. A variety of phages have been engineered to express compounds with the aim of increasing antibactericidal efficacy with a diverse set of targets. One common target for extracellularly acting pay-

loads are structural barriers such as bacterial biofilms and capsules, which provide a first line of defense against phage infection and can be breached by a variety of compounds. Another approach is employing payloads which are expressed by the host cell but also induce collateral damage on other bacterial species in the surrounding environment. One of the first bacteriophages engineered to have enhanced bactericidal activity using an extracellularly acting payload was a T7 phage modified to encode the polymerase Dispersin B, which showed significantly elevated levels of biofilm clearance in comparison to the wildtype phage [351]. Further phages have been engineered with payloads in this category, such as a quorum-quenching enzyme-expressing T7 phage with enhanced activity in mixed-species biofilms containing *Pseudomonas aeruginosa* [352] or an *E. amylovora* infecting phage Y2 and the integration of a depolymerase to enhance its potential as a treatment against fire blight [353]. Engineered phages encoding cell wall hydrolases have also shown success in expanding the range of bactericidal effect to other bacteria and/or enhancing killing efficacy [354, 355]. A number of phages expressing engineered payloads with intracellular effects have also been constructed in the past. These are typically armed with compounds which are capable of disrupting intracellular structures and functions, enhance antibiotic sensitivity, decrease virulence or confer sequence-specific toxicity to bacterial cells. One example are payloads interfering in the cellular SOS response in *E. coli* and thereby greatly increasing antibiotic potency [356] as well as the reversal of antibiotic resistance through integration of a prophage into the bacterial chromosome, which encodes for genes re-sensitizing the bacterium to specific antibiotics [357]. The arming of phages with payloads such as colicin-like bacteriocins (CLBs) have also shown great potential in increasing bactericidal efficacy [355]. CRISPR-Cas has also been employed as a phage-encoded payload to allow the discriminate targeting of virulent *S. aureus* while preserving avirulent strains [358]. This application specifically holds great promise for the sequence-specific targeting of bacterial pathogens exhibiting e.g., antibiotic resistance or specific virulence determinants [359]. Finally, the suitability

of phages as vaccines or drug delivery vectors in eukaryotes has been demonstrated in the past and shows great potential in therapeutic applications such as cancer therapy [360–362]. An overview of different engineered phages encoding payloads for enhanced antibacterial efficacy are shown in **Table 1**.

Table 3: Examples of phages engineered for enhanced antibacterial efficacy through genetic incorporation of heterologous payloads (adapted from [384]).

Phage	Host	Payload genes	Mode of action	Ref.
T7	*E. coli*	Biofilm-degrading enzyme (dspB); originating from *Actinobacillus actinomycetemcomitans*	Hydrolysis of polymeric -1,6-N-acetyl-d-glucosamine (PNAG) to disperse biofilms	[363]
Y2	*E. amylovora*	Depolymerase from *Erwinia* phage L1 (*dpoL1-C*)	Synergistic activity between the depolymerase degrading the exopolysaccharide capsule and lytic phage infection	[364]
T7	*E. coli*	Acyl homoserine lactones (AHLs) lactonase (*aiiA*) from *Bacillus anthracis*	Heterologous lactonase degrades AHLs from *Pseudomonas aeruginosa* and thus inhibit the formation of quorum-sensing dependent biofilms	[365]
PSA	*L. monocytogenes*	Endolysin from *Listeria* phage A511 (*ply511*)	Heterologous additional endolysin with broad activity allows targeting of non-host (phage PSA resistant) *Listeria* cells	[366]
A511	*L. monocytogenes*	lysostaphin-hexahistidine (*lst-his6*)	Hydrolysis of *S. aureus* peptidoglycan by heterologous phage-encoded lystostaphin produced inside *Listeria* cells	[354]

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Table 3: Examples of phages engineered for enhanced antibacterial efficacy through genetic incorporation of heterologous payloads (adapted from [384]). (Continued)

Phage	Host	Payload genes	Mode of action	Ref.
T7	*E. coli*	Leaderless bacteriocin lacticin Q (*lnqQ*); originating from *Lactococcus lactis*	Control of *B. coagulans* growth through heterologous lacticin Q released from *E. coli* hosts, increase of lytic activity against *E. coli* through interference of LnqQ with cytoplasmic membrane	[367]
T7	*E. coli*	Gene encoding antimicrobial peptide IDR (innate defense regulator) - 1018	Assist biofilm dispersal and confer enhanced bactericidal activity	[368]
M13	*E. coli*	Genes encoding antimicrobial peptides (cecropin, apidaecin) and protein toxin (*ccdB*)	Disrupt bacterial intracellular process including septum formation, DNA replication, protein production and DnaK activity	[369]
phi11	*S. aureus*	Gene encoding small acid soluble spore proteins	Sequence-independent binding and inactivation of bacterial DNA	[370]
M13	*E. coli*	addiction toxins (*gef* and *chpBK*)	Permeabilize host cell membrane (*gef*) and degrade mRNA (*chpBK*)	[371]
M13	*E. coli*	Gene encoding lethal catabolite gene activator protein	Disturb transcriptional regulation in adenyl cyclase-positive *E. coli* host	[372]
Pf3	*P. aeruginosa*	Restriction endonuclease (*BglIIR*)	Disrupt bacterial genome integrity	[373]

Continued on next page

Table 3: Examples of phages engineered for enhanced antibacterial efficacy through genetic incorporation of heterologous payloads (adapted from [384]). (Continued)

Phage	Host	Payload genes	Mode of action	Ref.
M13	*E. coli*	Restriction endonuclease (*BglIIR*) and modified phage λ S holin	Disrupt bacterial genome integrity and dissipate membrane potential	[374]
λ	*E. coli*	Dominant antibiotic-sensitive genes (*rpsL* and *gyrA*)	Abolish antibiotic resistance conferred by recessive alleles	[375]
M13-mp18	*E. coli*	Anbitiotic receptor (*ompF*) and diverse proteins targeting non-essential gene networks including: *lexA3* (repressor of SOS response), *soxR* (regulator of oxidative stress response); *csrA* (global regulator of glycogen synthesis and catabolism, gluconeogenesis, and glycolysis; repressor of biofilm formation)	Increase drug penetration and disturb bacterial gene networks, thereby enhancing bactericidal effect of antibiotics	[376]
λ	*E. coli*	Transcriptional repressor of the virulence factor (nondegradable 933 W.cl$^{\text{Ind}-}$)	Block lytic induction of the 933 W prophage, thereby neutralizing shiga-toxin (Stx2) production from enterohemorrhagic *E. coli* (*stx2* is encoded on 933 W lytic cassette)	[377]

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Table 3: Examples of phages engineered for enhanced antibacterial efficacy through genetic incorporation of heterologous payloads (adapted from [384]). (Continued)

Phage	Host	Payload genes	Mode of action	Ref.
NM1	*S. aureus*	RNA-guided Type IIA CRISPR-Cas9 targeting antibiotic resistance (*aph-3*; *mecA*) and virulence gene (*sek*); originating from *S. pyogenes*	Sequence-specific inactivation of target bacterial functions; immunize avirulent strains against horizontal transfer of plasmid-borne resistance/virulence genes	[378]
M13	*E. coli*	NA-guided Type IIA CRISPR-Cas9 targeting antibiotic resistance ($bla_{\text{SHV-18}}$, $bla_{\text{NDM-1}}$, gyr_{AD87G}) and virulence genes (*eae*); originating from *S. pyogenes*	Induce sequence-specific cytotoxicity, activate toxin-antitoxin systems, restore antibiotic sensitivity, and modulate bacterial consortia	[379]
M13; 80; 80α	*E. coli*; *S. aureus*	RNA-guided type VI CRISPR-Cas13a system targeting antibiotic resistance genes (*bla*, *mcr*, *mecA*); originating from *Leptotrichia shahii*	Sequence-specific killing of antibiotic-resistant bacteria	[380]
CD24-2	*Clostridioides difficile*	Exogenous bacterial genome-targeting crRNA (compatible with the endogenous type I-B CRISPR-Cas system in *C. difficile*)	Sequence-specific killing of target bacteria with enhanced efficacy as compared to wild-type parental phages	[381]

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Table 3: Examples of phages engineered for enhanced antibacterial efficacy through genetic incorporation of heterologous payloads (adapted from [384]). (Continued)

Phage	Host	Payload genes	Mode of action	Ref.
λ	*E. coli*	RNA-guided nuclease-deactivated Cas9 (dCas9); originating from *S. aureus*	Sequence-specific repression of target bacterial functions (fluorescence was used as a proof of concept)	[382]
M13	*E. coli*	Small regulatory RNAs (sRNAs) targeting transcripts of chloramphenicol acetyltransferase and kanamycin phosphotransferase	Knock down antibiotic resistance phenotype	[383]

1.7 Phage-based diagnostics

The detection of bacteria, and particularly the discrimination between pathogenic and non-pathogenic variants, ranges far beyond clinical applications and has significant implications in sectors such as food [385], agriculture [386] and even space exploration [387, 388]. In a clinical setting, early detection and correct identification of the underlying infection as well as the corresponding adaptation of the ensuing treatment regimens are crucial components for a positive treatment outcome [389]. Culture-based methods present the most basic form of pathogen detection and consist of the cultivation of bacterial culture *in vitro* on, e.g., selective nutrient media [390]. Other approaches include antibody-based immunoassays [391], lateral flow immunoassays (LFIA) [392], polymerase chain reaction (PCR) [392] and matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrometry [393]

Another popular approach is using phages to detect the presence of specific bacterial strains. The exceptional binding affinity and host specificity of phages make them ideally

suited as a bacterial detection method. Several different approaches exist, making use of quantifying the adsorption of phages to the cell surface, quantifying the production of phage encoded proteins or amplification of the phage genome [394].

One efficient method is the conjugation of compounds to the phage which allow subsequent detection or isolation of bacteria which have been bound by the phage receptor binding protein. These methods include electrochemiluminescent biosensors by conjugating phages with carboxyl graphene on to the surface of glass carbon electrodes [395], bioconjugated filamentous phages for the use in lateral flow assays [396] and the coupling of phages with magnetic beads to isolate various pathogens from complex matrices with high specificity [397–399]. Rather than using entire phage particles as conjugates, the same effect can also be achieved by using only the receptor binding proteins [400–403] or cell wall binding domains (CBD) of lysins and endolysin which convey the high specificity for phage binding [404–407].

Although methods based on the adherence of phages and phage-components to the bacterial cell wall often have the advantage of maintaining cell integrity, numerous infection-based methods exist and have been employed in the past with great success. The simplest approach is monitoring the infection by the number of released phage particles over time [408, 409]. Another method is the detection of compounds initially present intracellularly and released upon phage-mediated lysis, such as ATP and RNA [410, 411]. A powerful but typically more complex approach is the genetic engineering of phages to express reporter proteins which can be detected following phage-infection of the host bacterium. To this end, a number of different phages have been engineered in the past, employing a diverse set of payloads such as fluorescent proteins (fluorbacteriophages) [412–414], bioluminescent luciferases [415–418] and other proteins, including hydrolyzing enzymes such as beta-gal for calorimetric detection [419, 420]. Not only do these methods demonstrate rapid design, implementation, and detection of bacterial pathogens with high specificity, but by the use of highly sensitive molecules such as reporter luciferases only a very low

level of expression is already sufficient to detect a signal. This makes them ideal candidates for e.g. a companion diagnostic tool for phage therapy [421] or for the detection of slow-growing organisms such as *Mycobacterium tuberculosis* [422].

1.8 Phage engineering methodologies

A detailed overview of phage engineering, including it's background and methods, as well as speculations on the future of phage therapy using advances in synthetic biology and artificial intelligence, are given in **Manuscript I** and will only briefly be reiterated here.

Whereas prophages can be manipulated directly in the bacterial chromosome and subsequently isolated through induction, strictly lytic phages require specially adapted engineering methodologies to introduce genetic changes to the phage genome. Typically, this can be achieved via homologous recombination using a cytoplasmic editing template during phage infection. Although for the construction of reporter phages expressing e.g. a luciferase or fluorescent protein marker positive selection can be used [415, 423, 424], this is typically infeasible for non-reporter payloads or bacterial hosts with exceedingly low recombination frequency, such as *S. aureus* [425]. In these cases, making use of CRISPR-Cas systems as a means of counterselecting against wildtype phages and thereby enriching the relative abundance of recombinant phage drastically reduces the amount of screening needed to be done to isolate phages containing the genetic change of interest [424]. This has successfully been demonstrated in numerous studies [421, 426] (see also **Manuscript 2**). Another approach is the construction of genetically altered phage genomes via the assembly of synthetic genome fragments using methods such as transformation-associated recombination (TAR) or in vitro isothermal enzymatic assembly (Gibson assembly). The assembled DNA must then be reactivated, which can be achieved via transformation into e.g. a native host cell [347, 421, 426] or cell-wall deficient L-form bacteria [348, 418, 427] (see also **Manuscript 3**), as well as reactivation

using cell free transcription-translation systems [428–431]. One of the goals of this dissertation was to employ the above-mentioned engineering methods to engineer phage applications targeted against the major human pathogen *S. aureus*.

1.9 *Staphylococcus aureus*

Staphylococcus aureus was first identified by British surgeon Sir Alexander Ogston in 1880 in the abscess of a human knee joint [432, 433].*S. aureus* is a major human bacterial pathogen. It does not normally cause infection on healthy skin, although localized skin infections, typically resulting from small cutaneous lesions and subsequent *S. aureus* colonization can occur [434]. When allowed to enter the bloodstream or internal tissues, however, severe, and often life-threatening infections can develop [435].*S. aureus* is ubiquitous in the environment and one of the most common food-borne pathogens worldwide [436]. It is also a common human commensal and can be found on skin and mucous membranes, typically in the nasal area, in a significant portion of healthy individuals [437]. Up to 50% of adults are estimated to be colonized with *S. aureus*, with 15% showing persistent colonization in the anterior nares. Furthermore, subpopulations such as health care workers, intravenous drug users, hospitalized patients and immunocompromised people have been shown to have *S. aureus* colonization rates ranging up to 80% in frequency [438]. Infection can occur through primary colonization followed by migration, colonization, and infection of other body sites. The second mode is through direct contact with colonized or infected individuals as well as transmission via fomites [439]. *S. aureus* is known for its wide array of clinical manifestations, including bacteremia, infective endocarditis, skin and soft tissue infections (e.g., impetigo, folliculitis, furuncles, carbuncles, cellulitis, scalded skin syndrome, and others), osteomyelitis, septic arthritis, prosthetic device infections, pulmonary infections (e.g., pneumonia and empyema), gastroenteritis, meningitis, toxic shock syndrome, and urinary tract infections [440]. Al-

though direct tissue invasion is the most common form of *S. aureus* induced disease, toxin-mediated disease, as is the case for toxic shock syndrome, *staphylococcal* scalded skin syndrome or *staphylococcal* food poisoning, can also occur [440]. Community- as well as hospital-acquired infections are pervasive and often associated with numerous additional challenges stemming from the emergence of MDR variants such as methicillin-resistant (MRSA) and vancomycin-resistant (VRSA) *S. aureus* [438].

Staphylococci are Gram-positive and typically grow in cocci shaped, grape-like clusters. The etymology combines the prefix "staphylo-" (from Ancient Greek: 'bunch of grapes'), and suffixed by the Modern Latin: coccus, 'spherical bacterium' (from Ancient Greek: 'grain, seed, berry') [441]. The species classification and entymology of *S. aureus* stems from the characteristic golden or yellow color (from Latin aurum: 'gold') [441]. *S. aureus* can grow both aerobically or facultative anaerobically in up to 15% salt concentrations and a temperature range from 15°C to 45°C [442]. Different Staphylococcus species can be biochemically identified via an array of assays, including catalase positive (all pathogenic *Staphylococcus* species), coagulase positive (including *S. aureus*), novobiocin sensitive (*S. saprophyticus*) and mannitol fermentation positive (*S. epidermidis*) [443]. Gram-positive bacteria are characterized by a significantly thicker peptidoglycan cell wall compared to their Gram-negative counterparts [444]. The glycan strands are cross-linked by peptide bridges which provide the structural integrity of the cell wall and are interspersed with teichoic acids and wall-associated surface proteins [445]. A number of immune evasion mechanisms of *S. aureus* exist, including the production of an antiphagocytic capsule, sequestering of host antibodies or antigen masking by Protein A, biofilm formation, intracellular survival, and blocking chemotaxis of leukocytes [446, 447]. A further important factor contributing to virulence are superantigens (e.g. TSST-1 or toxic shock syndrome toxin 1) which contribute to the manifestation of diseases such as infectious endocarditis, sepsis and toxic shock syndrome [448, 449]. Finally, communication via quorum sensing and the associated formation of more complex local

population structures such as biofilms further contribute to virulence and immune evasion and are particularly frequent in prosthetic joint- or medical implant infections [450]. MRSA strains have been shown to carry the *mec* gene cassette (SCCmec) integrated into the chromosome and, depending on the specific variant, can confer resistance to multiple antibiotics [451]. *mec* encodes for penicillin-binding protein (PBP) 2A, a class of protein responsible for the synthesis of the peptidoglycan backbone by facilitating the reactions crosslinking neighboring peptidoglycan strands [452]. PBP-2A is a variant of PBP which shows much lower affinity to beta-lactams when compared to other PBP variants found in e.g. methicillin-sensitive *S. aureus*, and cell-wall synthesis is therefore not effectively inhibited by antibiotics such as methicillin, nafcillin, oxacillin and cephalosporins [452].

Detection of a *S. aureus* infection is typically achieved via routine culture assays (e.g. from the blood, sputum), although quantitative real-time PCR for 16S rRNA genes is sometimes necessary [438]. Often, additional characterization is necessary to discriminate pathogenic from commensal *S. aureus* variants, the latter of which are frequently members of the normal skin and nasal flora [438]. First-line treatment is typically in the form of penicillin for methicillin-sensitive *S. aureus* strains, and vancomyin for MRSA infections [453]. However, alternative treatment regimens are sometimes necessary, such as fluid-replacement for toxin-mediated illness or removal of a medical implant which shows evidence of *S. aureus* biofilm formation [438, 454]. Although alternative strategies such as vaccines have remained elusive thus far [455], phage therapy provides an ideal alternative to treat *S. aureus* infections, particularly for antibiotic resistant strains. Several reports of successful treatment of *S. aureus* via phage therapy already exist. These cases demonstrate multiple successful applications in *S. aureus*-caused bone and joint infections, including the treatment of diabetic foot ulcers [456], distal phalanx osteomyelitis [457] and a number of other bone and joint infections [458, 459]. Promising results for chronic skin and soft tissue infections [460] as well as heart and pulmonary infections [461–463] have also been achieved. A number of past and on-going clinical

trials to evaluate the efficacy and safety of phage therapy targeted against *S. aureus*-induced wound infection, chronic rhinosinusitis and prosthetic joint infections also show promising results [464–468].

1.10 Aim of the thesis

The general scope of the thesis is to combine synthetic biology and genetic engineering methodologies to demonstrate the potential of genetically engineered phages targeted against *S. aureus*. The thesis comprises 5 manuscripts. Manuscript 1 (Section x), *Enhancing phage therapy through synthetic biology and genome engineering*, gives a comprehensive overview of state-of-the-art engineering methodologies and the different ways they can be applied to enhance the therapeutic efficacy of naturally occurring phages.

In manuscript 2, *Genetically engineered Kayvirus K::nluc enables rapid detection of viable Staphylococcus cells in blood and milk.*, we demonstrate the use of homologous recombination (HR) and CRISPR counterselection to engineer a large, lytic *S. aureus* phage with broad host range to express a nanoluciferase reporter gene. We further demonstrate the application of the engineered reporter phage to detect a wide variety of vancomycin-resistant *S. aureus* clinical isolates in complex media such as human whole blood and bovine raw milk.

An alternative to HR-based engineering is reactivation, or "rebooting", of synthetic phage genomes in cell wall-deficient L-form bacteria. The cultivation of such L-form bacteria, the design and assembly of a synthetic phage genome, as well as the rebooting of said genome in L-form bacteria is demonstrated in Manuscript 3, *Genetic manipulation of bacteriophages in L-form bacteria.*

Conventional practice for integrating payloads intended to be expressed during the infection cycle is to insert the payload in question behind the bacteriophage major capsid protein to ensure high expression levels common for this genomic region. Manuscript

4, *Computational prediction of high-expression loci on the phage genome for targeted introduction of payload genes*, aims at determining alternative sites for the introduction of payload genes. By using a machine learning approach optimized for the detection of phage-encoded promoters from genomic data, we determine sites with potentially high expression levels based on the density and machine learning-based predictive score. We subsequently attempted to experimentally validate the expression levels of payload genes inserted at those loci.

Manuscript 5, *Genetic engineering of lytic S. aureus phage for the expression of antimicrobial payloads*, screens a large panel of antimicrobial payloads, which have previously been shown to exhibit activity against *S. aureus*, for viability and potential enhanced killing efficacy.

Overall, the thesis gives insights into techniques, applications and challenges associated with the genetic engineering of *S. aureus*-infecting phages for enhanced therapeutic potency.

1.10.1 Specific contribution by the doctoral candidate

All laboratory experimentation was conducted by the doctoral candidate or by undergraduate students under the direct supervision of the doctoral candidate. The bacterial strains Rev2L and SuL27 used in the manuscript *Genetic manipulation of bacteriophages in L-form bacteria* were generated and provided by co-authors SK and SM, respectively. Concerning the manuscript, *Enhancing phage therapy through synthetic biology and genome engineering*, the doctoral candidate's specific contribution lies in conceptualization, writing of the introduction and outlook chapters as well as design and visualization of all figures. The first authorship is shared equally by the doctoral candidate and co-author BL. All other manuscripts were written in full- and are first-authored by the doctoral candidate and revised by SK, albeit the "Materials and Methods" section of Manuscript IV, parts of which were written by EH.

Chapter 2

Manuscripts

2.1 Manuscript I: Enhancing Phage Therapy through Synthetic Biology and Genome Engineering

Specific contribution by the doctoral candidate

The doctoral candidate contributed by conceptualization of the manuscript, visualization (design and implementation of figures and graphical abstract), writing (first draft for introduction, outlook and conclusion) and review and editing of the manuscript.

Enhancing Phage Therapy through Synthetic Biology and Genome Engineering

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Highlights:

- Synthetic biology enables efficient phage genome engineering.
- Phages with tunable host range and antimicrobial payload delivery enhance efficacy.
- Phage design principles may be guided by computational approaches in the future.

Abstract

The antimicrobial and therapeutic efficacy of bacteriophages is currently limited, mostly due to rapid emergence of phage-resistance and the inability of most phage isolates to bind and infect a broad range of clinical strains. Here, we discuss how phage therapy can be improved through recent advances in genetic engineering. First, we outline how receptor-binding proteins and their relevant structural domains are engineered to redirect phage specificity and to avoid resistance. Next, we summarize how phages are reprogrammed as prokaryotic gene therapy vectors that deliver antimicrobial "payload" proteins, such as sequence-specific nucleases, to target defined cells within complex microbiomes. Finally, we delineate big data- and novel artificial intelligence-driven approaches that may guide the design of improved synthetic phage in the future.

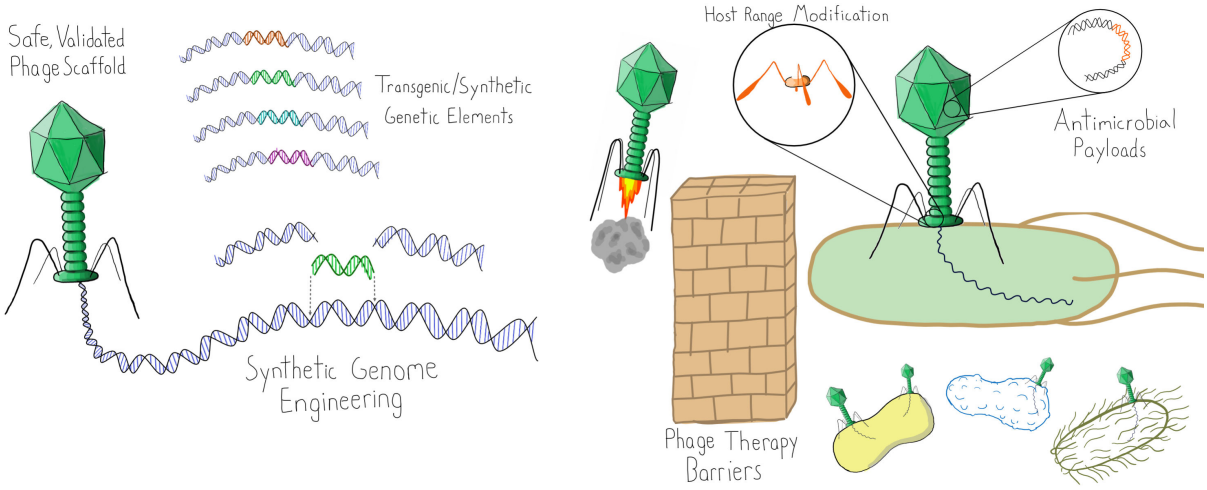

Figure 1: Graphical abstract

Introduction

In recent decades, the widespread and often poorly regulated use of antibiotics in medicine and agriculture has escalated the emergence of antibiotic-resistant pathogens [1]. The increasing burden placed on health care systems by these multidrug-resistant (MDR) microorganisms, alongside the simultaneous decline in pharmaceutical companies developing and stockpiling novel antibiotics [2], presents a dire threat to continued human prosperity [3]. As a result, there is rekindled interest in the use of phages as potential antimicrobial therapeutics [4], underlined by a series of recent successful compassionate use cases and upcoming randomized clinical trials (e.g. NCT03808103 and NCT04191148) [5,6]. However, *in vitro* and clinical data suggest that the efficacy of phage therapy using natural phage may be limited due to the rapid selection of phage resistant bacteria [6,7], due to immunogenicity as a consequence of prolonged treatment [8], and also because the host ranges of natural phage isolates rarely cover all clinically relevant pathogenic strains. Advances in synthetic biology, where engineering principles are utilized to design biological systems from interchangeable parts, and the increasing pace of phage discovery may enable the augmentation of natural phage properties to overcome many of these shortcomings. Recent studies describing naturally-evolved phage systems to broaden host range [9] and evade host defenses such as CRISPR-Cas [10,11], suggest that phage genomes contain a treasure trove of natural antimicrobial mechanisms and products to add to the synthetic biology toolbox. Here, we review the phage engineering techniques used to integrate such components into designer phages that possess enhanced properties over their naturally occurring counterparts, such as altered/broadened host ranges and the ability to transduce therapeutic payload genes to defined targets within the microbiota. We also give a speculative outlook on the future use of artificial intelligence and machine learning approaches to design the next generation of phage-based therapeutics.

Phage genome engineering methods

The stable integration of temperate phage genomes into host chromosomes results in a resident prophage, and generally enables virus manipulation via the same methodologies as those for bacterial genomes. In contrast, strictly lytic (virulent) phages require specialized genome engineering methods, which can all be classified into two broad conceptual groups: i) homologous recombination (HR), and ii) genome rebooting, i.e., the activation of exogenously assembled, synthetic phage DNA.

Homologous recombination

Recombination-based approaches (**Figure 1A**), where the phage genome undergoes HR-driven allelic exchange with a cytoplasmic editing template during infection, are the most commonly used methods of phage genome engineering. However, with respect to strictly lytic phage, these methods are limited by low natural recombination frequencies and require extensive screening to obtain progeny phages with the desired mutations [12-14].

Recombination efficiency has been significantly improved by co-opting the natural recombination systems of temperate phages in a method termed recombineering. Expression of phage recombination proteins (e.g. lambda Red and Rac RecE/RecT) within the recombination host protects the editing template from degradation and facilitates annealing with the injected phage genome (in vivo recombineering), thereby increasing recombination frequency and reducing the homology arm length requirement [15]. The identification of RecE/RecT homologs in mycobacteriophage Che9c expanded these techniques to Gram-positive bacteria and enabled the bacteriophage recombineering of electroporated DNA (BRED) technique, in which the phage genome and editing templates are co-electroporated into recombineering hosts [16].

HR-based editing techniques have also been improved through the use of positive and negative selection of progeny phages. The insertion of reporter genes (e.g. luciferase or fluorescent proteins) or phage-specific marker genes (e.g. *trxA* or *cmk* in coliphages)

into phage genomes facilitates rapid positive selection of recombinant phages, despite low recombination frequencies [13,17,18]. Recently, bacterial CRISPR-Cas systems have been adapted as a mechanism for the negative selection of unmodified phage progeny, effectively enriching rare mutants from recombinant phage lysates. To date, type I-E, II-A, and III-A CRISPR systems have been used to select for recombinant phages targeting Gram-negative and Gram-positive hosts [19].

Genome rebooting

To address the problem that phage gene products may be toxic to their bacterial host, synthetic methods for genome assembly outside of the natural bacterial hosts have been developed. These techniques rely on the assembly of small to medium-size DNA fragments into full-length phage genomes through transformation-associated recombination (TAR) or *in vitro* enzymatic assembly (Gibson assembly) followed by transformation into competent bacterial hosts for rebooting and assembly into mutant phage particles (**Figure 1B**). Phage genome assembly from synthetic DNA fragments enables flexible engineering to introduce mutations, deletions, or insertions at any genomic locus, scales easily for genetic library construction, and eliminates the need to select against wild-type sequences.

Jaschke et al. [20] first demonstrated the efficacy of such approaches to reconstruct and archive the ϕX174 phage genome in yeast. The small 5.4 kb ϕX174 genome could be assembled directly from PCR products and synthetic fragments into a yeast artificial chromosome through TAR, released through restriction enzyme digestion, and rebooted in *Escherichia coli* cells. Ando et al. [21] utilized similar approaches to modulate the host range of several T7-like phages to target various Gram-negative pathogens (see below). Due to the requirement for high transformation efficiency and access to the *E. coli* molecular machinery, these approaches were initially limited to phages infecting Gram-negative hosts. Recently, cell wall-deficient L-form bacteria have been developed

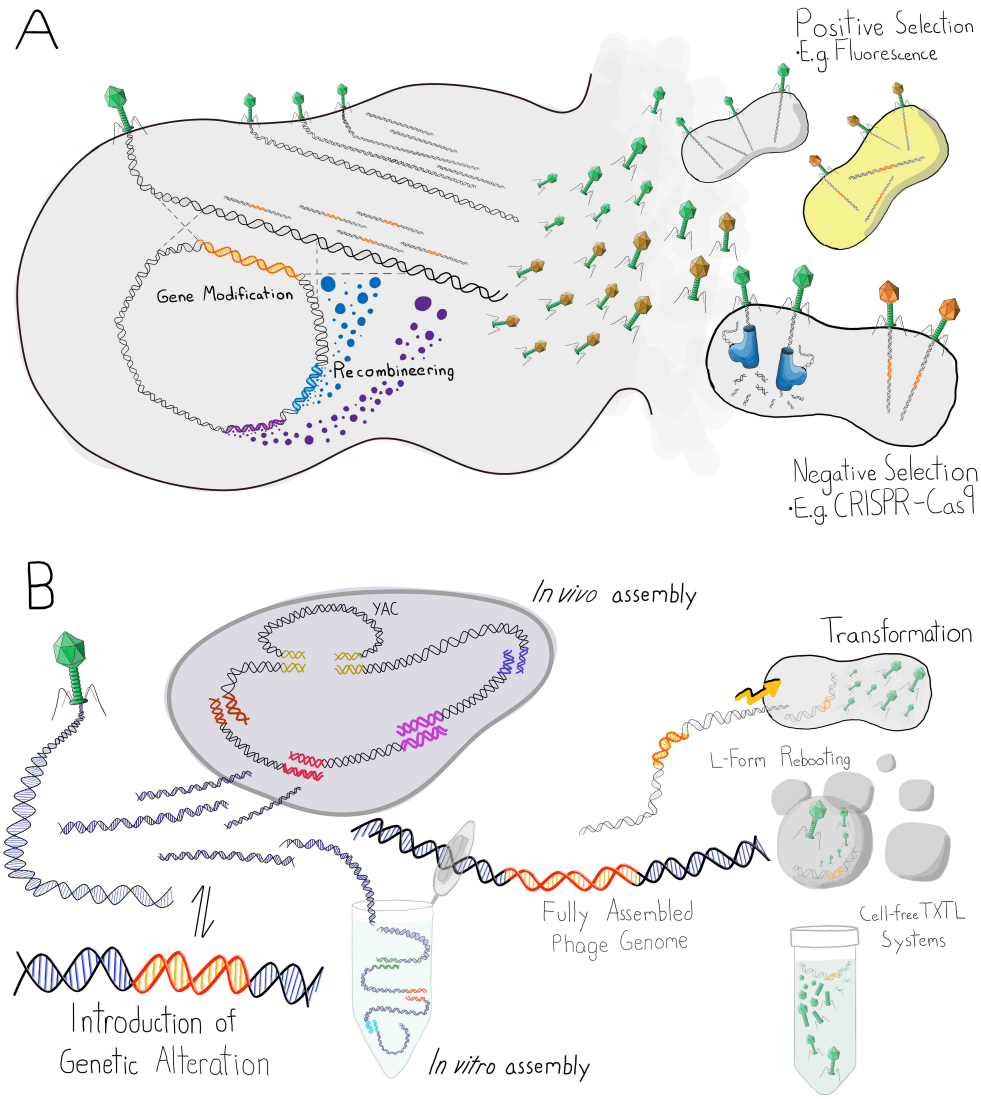

Figure 2: Phage genome engineering methods.

(A) Homologous recombination is often used to engineer phage genomes. In recombineering, heterologous recombination proteins are expressed within the cell, thereby increasing recombination frequency and protecting the recombination template from intracellular degradation. Both positive selection (e.g. fluorescence markers) and negative selection (e.g. CRISPR-Cas9 targeted against the wild-type phage) can be used for downstream selection of engineered phage particles. (B) Phage genomes can also be assembled from synthetic DNA fragments to introduce desired genetic alterations. Both *in vivo* (e.g., TAR-cloning) and *in vitro* (e.g., Gibson) assembly techniques are used. The fully assembled phage genome is rebooted to produce fully functional phage particles either in Gram-negative bacteria following electrotransformation, or using cell-free transcription-translation (TXTL) systems. Phage genomes of Gram-positive bacteria are rebooted in cell-wall deficient L-form bacteria after PEG-assisted transformation.

as effective Gram-positive phage rebooting hosts following genome transformation using polyethylene glycol. Kilcher et al. [22] demonstrated cross-genus rebooting of various Gibson-assembled and wild-type phage genomes in L-form *Listeria monocytogenes*, suggesting that this approach may be broadly applicable for phages infecting Gram-positive bacteria. This technique was subsequently used to construct reporter phages [23] and to modify the host-range of a *Listeria* phage (see below) [24].

New synthetic biology techniques to reboot phage genomes outside host cells eliminate the need for DNA transformation and enable the synthesis of phages infecting unknown or undomesticated hosts. High yields of self-assembling MS2, T4, T7, and ϕX174 phage particles have been generated in a test tube from a small quantity of phage DNA and optimized *E. coli* extracts, via cell-free transcription-translation (TXTL) systems [25-27]. Although genetic engineering has in the past been limited to phages infecting well-studied laboratory hosts, recent high-throughput screens for recombination protein homologs [28] and the development of TXTL systems [29] from additional bacteria is expected to expand future phage engineering to new bacterial hosts (e.g. *Vibrio*, *Streptomyces*, *Bacillus*, and *Pseudomonas* species).

Designer phage applications

The techniques outlined above have been used to create genetically modified phages for use in bacterial diagnostics, therapeutics, and drug delivery [30,31]. Below, we will highlight efforts to engineer designer phages with tunable host range and enhanced payload delivery to develop the next generation of phage therapy.

Programming host specificity

Phage host specificity is a double-edged sword: although phages can selectively infect and kill host bacteria within complex microbial communities, individual phages often lack

sufficient host range to target all strains responsible for clinical infections [9]. Multi-phage cocktails can target various pathogenic strains and prevent the proliferation of phage-resistant mutants; however, the isolation and characterization of the constituent phages is time-consuming and requires laborious regulatory approval [32]. To ameliorate these limitations, synthetic biologists are now implementing design principles from the natural host range expansion mechanisms of broad-host-range phages for the scalable synthesis of modular phages, creating a tunable host range based upon previously validated phage scaffolds (**Figure 2A**).

The primary host-range determinants are receptor-binding proteins (RBPs) located either at the distal end of the phage adsorption apparatus (the baseplate), or at the tip of the tail fibers, which mediate the interaction with carbohydrate or protein receptors at the host cell surface. Broad-host-range phages have evolved strategies to expand or switch host range, including: i) RBP allele and domain exchange, ii) targeted RBP diversification in hypervariable regions, and iii) polyvalent RBP virions [9].

Rational engineering of phage host range has so far been accomplished by tail fiber exchange via HR between closely-related phages in the T2, T4, and T7 families [12,14,33-37]. Ando et al. expanded these techniques to a broad range of T7-like phages using a yeast-based genome rebooting strategy in order to create phage scaffolds with modular exchange of those tail components dictating the host range [21]. Whereas exchanging complete or partial T7 phage scaffold tail fibers (gp17) with those of closely-related phages effectively modulated host range, the creation of viable phage hybrids between *Escherichia* phage T7 and *Klebsiella* phage K11 required the exchange of the complete tail apparatus (gp11, 12, and 17), thus also demonstrating certain limits of RBP modularity.

Most mature RBPs are involved in multiple protein-carbohydrate and protein-protein interactions with the host cell receptor as well as with structural phage tail proteins. These interactions are required to maintain structural integrity, to mediate receptor

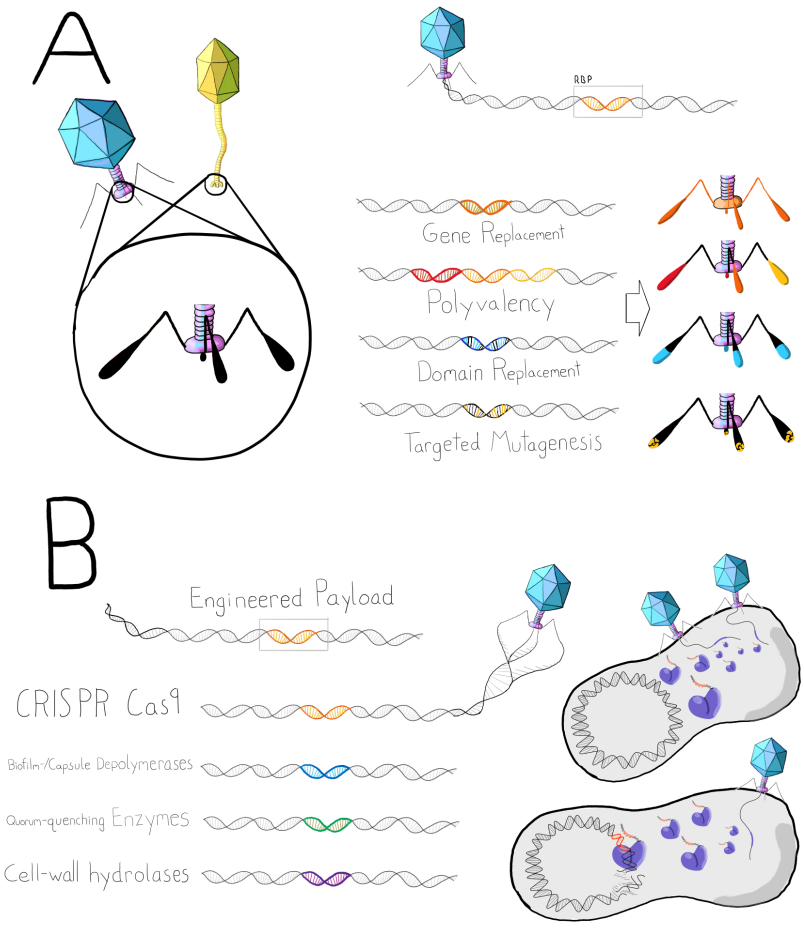

Figure 3: Designer phage applications.

(A) Host specificity can be (re-)programmed by altering the phage receptor binding modules. Genetic alterations of these modules may be introduced at different scales, ranging from full gene replacements (e.g. tail or RBP swapping) to individual point mutations (e.g. amino acid exchanges in distal loops at the host-RBP interface). (B) Payload engineering refers to "arming" the phage with genes and properties aimed at increasing antimicrobial activity. For example, including CRISPR-Cas9 as a genetic payload can introduce intracellular sequence-specific toxicity whereas phage-encoded biofilm depolymerases target extracellular bacterial polymers in the vicinity of the lysed host cell.

recognition, and to initiate and orchestrate receptor-mediated structural rearrangements. It is therefore not an easy task to modify RBPs without losing infectivity. Domain shuffling techniques have been developed to create chimeric RBPs by exchanging the globular receptor-binding domains located in the C-terminal domain (CTD) of phage tail fibers, while leaving the N-terminal domain required for interactions with the phage tail intact [21,24,33,34].

Recently, high-throughput RBP diversification strategies have been implemented to screen for phage RBP mutants with shifted or expanded host ranges. Yehl et al. [38] used a targeted mutagenesis approach inspired by antibody engineering to generate functional diversity within unstructured loops located at the RBP-host cell interface of *Enterobacteria* phage T3. The resulting synthetic "phagebody" libraries (107 variants) contained individual phages with expanded host range and suppressed the evolution of phage resistance. Yosef and co-workers integrated tail/RBP allelic exchange and iterative cycles of targeted mutagenesis to engineer the host ranges of phage particles for optimized DNA transduction and payload delivery (see below) into new bacterial targets [39]. These techniques rapidly increase RBP diversity at a pace unachievable through natural evolution and help to create synthetic phage populations one step ahead of their bacterial hosts in the co-evolutionary arms race.

Dunne et al. combined RBP and lysogeny engineering to reprogram the temperate, narrow-range *Listeria* siphovirus PSA into a strictly lytic phage with broadened host specificity [48]. Akin to the T7 phagebody approach, the PSA host range was shifted via RBP diversification using targeted mutagenic PCR and subsequent rebooting of genome libraries in *Listeria* L-form cells. Additionally, a second rbp allele targeting a different *Listeria* serovar was inserted into the PSA genome to generate a synthetic phage featuring polyvalent host cell binding. Finally, the elucidation of the RBP CTD crystal structure enabled the structure-guided design of chimeric RBPs. Globular, C-terminal affinity domains identified in sequenced *Listeria* genomes were swapped at conserved

helical bundle motifs connecting the CTD to the baseplate. This approach generated viable chimeric phages, whose modified and expanded host-ranges were predicted through globular domain phylogeny and the glycotype of the donor lysogen.

It is evident that all these novel rational design principles depend on a detailed understanding of the molecular interactions dictating individual phage-host interactions. Thus, high-throughput tools to identify phage RBPs and their conserved structural domains are required to rapidly engineer modular phages at sufficient scale for therapy.

Delivering Cas nucleases as antimicrobial payloads

Besides engineering host range, phage antimicrobial activity can be enhanced or modulated through the *in situ* production of heterologous proteins, often described as genetic "payloads" (**Figure 2B**) [30]. For example, phages have been engineered to deliver biofilm- and capsule-depolymerases [40,41], quorum-quenching enzymes [42], and cell wall hydrolases with cross-genus lytic activity [22]. Infected cells produce and release these proteins upon host cell lysis where they act on target cells or substrates in their vicinity. Another group of payload proteins that have recently gained much attention are CRISPR-Cas nucleases. Here, the phage serves as a target cell-specific vector to deliver a programmable Cas nuclease toxin, effectively creating a nucleotide sequence-specific antimicrobial. Two initial landmark studies describe the use of non-replicative phagemids that target antimicrobial resistance- or virulence genes in *E. coli* [43] and *Staphylococcus aureus* [44], using sequence-specific crRNA and the type II-A effector nuclease Cas9. Phagemid delivery enabled selective removal of resistant or virulent bacterial sub-populations and can be employed to immunize against plasmids containing antibiotic-resistance genes. The same pathogens were later also targeted by engineered temperate phages delivering Cas9 or Cas3 (type I-E) effectors [45,46]. Very recently, Selle et al. presented a slightly modified approach that makes use of conserved type I-B CRISPR-Cas systems present in most *Clostridium difficile* genomes [47]. Based on

the temperate phage ϕCD24-2, the authors engineered a set of phage derivatives that delivered host-targeting crRNA as a toxin and/or feature genomic deletions that convert the phage lifestyle from temperate to virulent. Both life-style conversion and crRNA-mediated genome targeting improved killing *in vitro* and in a *C. difficile* mouse infection model. Similarly, Lam et. al used engineered, Cas9-delivering M13 phagemids to selectively deplete one of two *E. coli* strains competitively colonizing the gut of mice [48]. These two studies highlight the potential applicability of engineered phage therapy for gastrointestinal targeting and *in vivo* microbiome engineering.

Outlook and conclusions

The advent of big data and the pioneering of novel machine learning (ML) methodologies constitute a major technological advancement in biological and medical research. Whereas the rational engineering efforts described above have been modeled on natural phage systems, synthetic biology approaches integrating recent advancements in machine learning (ML) may further boost phage therapy as a viable clinical treatment.

Research combining 'omics with ML-powered bioinformatics is already being widely used in cancer and other biomedical fields [49]. These methods have thus far been limited in phage therapeutics, barring a handful of publications [50-53], an inattention most likely due to the scarcity of data available from online resources. As it stands, the most abundant dataset for any class of phage is an *Actinobacteriophage* collection comprising more than 3400 complete genome sequences (The *Actinobacteriophage* Database; URL: https://phagesdb.org). One trending approach is to utilize phage and bacteria whole-genome sequences from such databases, along with corresponding experimentally determined host-range information, to predict virus-host interactions (**Figure 3A and B**). While the *Actinobacteriophage* collection covers a sequence space of sufficient size for ML applications, the vast majority of these phages were isolated on a single bacterial species

(*Mycobacterium smegmatis*), which drastically reduces its applicability. Generally, comprehensive and reliable host-range data is difficult to obtain as it requires labor-intensive experimentation and standardized protocols and scoring systems that allow comparing host range data from different laboratories. Negative interaction data (i.e., bacterial hosts not infected by a given phage) is particularly underrepresented, a limitation that could be solved only by implementing complex data augmentation methods [54] or by making unvalidated assumptions regarding phage specificity.

Just as sequence-based, ML-driven phage research is on the rise, so are the inferences and predictions of biological processes being made on the basis of structural data. In addition to utilizing host-range information to infer phage sensitivity based on predicted protein-protein interactions [52], recent advances in glycan research are similarly promising. Glycans make up a large portion of the bacterial cell surface and often function as phage attachment ligands and receptors. Two recent studies by Bojar et al. [55,56] use natural language processing to create a comprehensive database of glycan structures with associated phage-binding information readily available and infer the evolutionary relationships of the bacterial species [57,58]. This opens an interesting angle for AI-driven phage research. Analogous to using host-range information as a classification label, these types of experimental data could very well serve as structural input data to predict phage-host interaction (**Figure 3C**).

To fully develop the untapped potential of phage therapy, the authors firmly believe that an interdisciplinary approach is required. Besides genome engineering, synthetic biology, structure-guided design, and machine learning, this includes a plethora of other fields such as drug formulation and administration, pharmacokinetics/-dynamics, and immunology. When coupled with a non-prohibitive regulatory framework for the manufacturing and application of phage-based biologics, this integrative approach paints a carefully optimistic picture of a future with targeted phage therapeutics effective against multidrug-resistant pathogens.

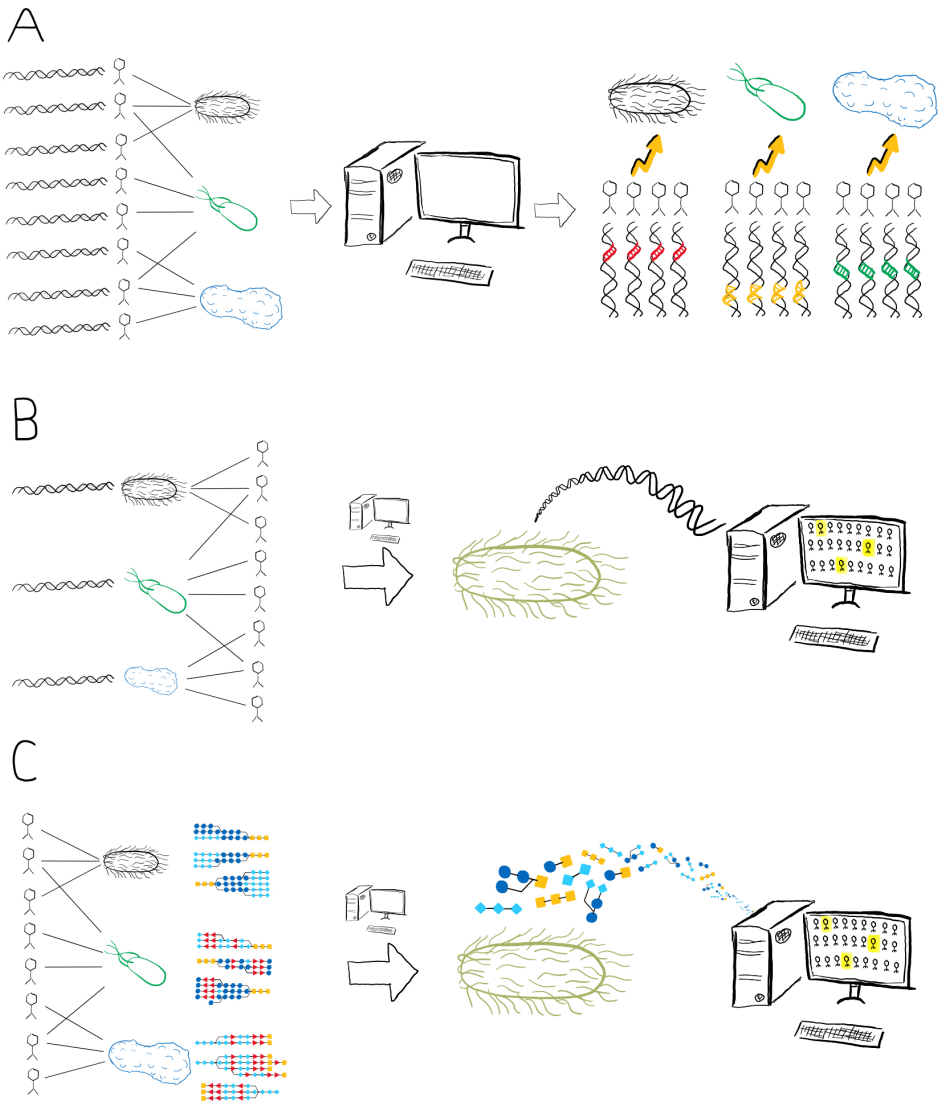

Figure 4: Artificial intelligence-driven phage research.
The figure outlines different applications of machine learning (ML) and artificial intelligence (AI) to phage engineering. (A) Given a novel phage sequence, a ML model can by trained on phage whole-genome sequences as features and corresponding host range information as classification labels. This approach could enable the prediction of phage-host interactions and provide insights into potential genetic elements dictating host range. (B) Similarly, by combining bacterial whole-genome sequences with information on phage infectivity, an AI algorithm could be trained to identify a selection of phages likely to infect a novel pathogen. (C) By associating bacterial surface glycans, i.e., using (partial) surface glycan structures as input data, with information on phage infectivity, the model could predict phage binding to a host cell surface.

Conflict of interest statement

T.K.L. is a co-founder of Senti Biosciences, Synlogic, Engine Biosciences, Tango Therapeutics, Corvium, BiomX, and Eligo Biosciences. T.K.L. also holds financial interests in nest.bio, Ampliphi, IndieBio, MedicusTek, Quark Biosciences, and Personal Genomics. M.J.L holds financial interests in Micreos.

Acknowledgements

We would like to thank Karen Pepper for critical review and revising the manuscript. Funding: S.K. is funded by the Swiss National Science Foundation (grant PZ00P3_174108). B.R.L is funded through the National Institutes of Health [grant 5-R33-AI121669-04]. T.K.L. is supported through the National Institutes of Health [grants 1DP2OD008435, 1P50GM098792, 1R01EB017755], the Defense Threat Reduction Agency [grant HDTRA1-14-1-0007], and the Institute for Soldier Nanotechnologies [contract W911NF-13-D-0001].

Author contributions

Bryan R. Lenneman: Conceptualization, Writing – Original Draft, Review & Editing. **Jonas Fernbach**: Conceptualization, Visualization, Writing – Original Draft, Review & Editing. **Martin Loessner**: Review & Editing, Funding acquisition. **Timothy K. Lu**: Review & Editing, Funding acquisition. **Samuel Kilcher**: Conceptualization, Writing – Original Draft, Review& Editing, Funding acquisition.

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2.2 Manuscript II: Genetically engineered Kayvirus K::*nluc* enables rapid detection of viable *Staphylococcus* cells in blood and milk.

Specific contribution by the doctoral candidate

All laboratory experimentation was conducted by the doctoral candidate or by undergraduate students under the direct supervision of the doctoral candidate. The manuscript was written in full- and first-authored by the doctoral candidate. Figures were designed and created by the doctoral candidate. The manuscript was revised by SK.

Genetically engineered *Kayvirus* K::*nluc* enables rapid detection of viable *Staphylococcus* cells in blood and milk.

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Keywords: *Staphylococcus aureus*, bacteriophage, genetic engineering, antibiotic resistance

Abstract

Staphylococcus aureus is an important opportunistic pathogen, causing a variety of diseases that can be difficult to treat due to resistance to methicillin, vancomycin, and other antimicrobials. Bacteriophages offer an ideal alternative to target pathogens where conventional drugs fail. The antimicrobial efficacy of phage therapeutics can be further improved through genetic engineering. For *S. aureus*, members of the *Twortvirinae* subfamily of strictly lytic, broad host-range phages are currently the most promising therapeutic candidates. However, due to their large genome sizes, these phages are notoriously difficult to engineer. In this study, we used *Twortvirus* K as a model and developed a simple and efficient phage engineering platform that is based on homologous recombination and CRISPR-Cas9-assisted counterselection. This tool was employed to engineer a nanoluciferase (*nluc*)-encoding reporter phage (K::*nluc*), which was subsequently developed into a rapid, bioluminescence-based tool for the detection of viable *Staphylococcus* cells. Independent of their resistance-profile, 100% of tested clinical *S. aureus* isolates emitted bioluminescence as a response to K::*nluc* challenge. This diagnostic assay was also adapted to complex matrices such as human whole blood and bovine raw milk to mimic *S. aureus* detection in bacteremia and bovine mastitis. Beyond reporter phage-based diagnostics, our engineering technology opens avenues for design and engineering of therapeutic *Twortvirinae* phages to combat drug-resistant *S. aureus* strains.

Introduction

The prevalence of multi-drug resistant (MDR) pathogens among the human population has undergone a steady increase in recent decades [1-3], with the WHO speculating the mortality of MDR-associated deaths to supersede even that of cancer by 2050 [4]. Although the multitude of antibiotics present on the market has greatly aided treatment of widespread, debilitating disease and experienced a marked increase over the last century [5], the ease of access to over-the-counter antibiotics, as well as over-prescription and use in the medical and agricultural fields, has fueled the emergence of a large number of MDR pathogens [6]. Bacteriophages and bacteriophage-derived proteins (endolysins) are promising alternatives to conventional antibiotic treatment and have increasingly been postulated as a main force to combat the antibiotic resistance crisis in years to come [7-9]. Numerous recent case reports demonstrated the successful application of phages to treat MDR infections [10-12] and rapid advances in synthetic biology and genetic engineering have allowed for the design, development and application of genetically altered phage variants with enhanced clinical potential [11, 13, 14].

Staphylococcus aureus is one of the most prevalent human pathogens, is known to colonize up to 50% of humans, and can result in severe disease such as pneumonia, respiratory-, surgical- and cardiovascular infections, as well as nosocomial bacteremia [15, 16]. Bacteremia alone has an estimated annual incidence of up to 50 cases/100 000, with 10% to 30% of these patients dying as a direct result of the infection [17]. Furthermore, resistance emergence in *S. aureus* isolates against a number of different antibiotics has been reported in literature. Methicillin (MRSA) and Vancomycin (VRSA) resistant *S. aureus* are two of the variants of greatest concern, posing as some of the most prevalent MDR, hospital-acquired infections and presenting a great burden and increased threat to health-care systems worldwide [18-20].

The use of bacteriophages to treat bacterial infections has experienced a surge in recent

years. Furthermore, novel methods for genetically modifying phages to achieve enhanced functionality are constantly being researched. Several genetically engineered bacteriophages, targeting a variety of bacterial pathogens, have previously been reported [13, 14, 21-24]. By rational selection and engineering of the phage backbone, various characteristics, such as altered host specificity, can be tuned for a specific clinical application. In the past, similar methods have been used to switch phages to an entirely new host [13, 21, 22], alter the infection cycle for clinical suitability [11] and integrate antimicrobial payloads to be delivered to sites of infection [14].

Kayviruses, such as phage K and closely related K-like phages, are lytic *myoviruses* with large genome size and exhibit a wide host range across *S. aureus* [25]. Previous attempts to engineer phage K using synthetic biology-based approaches have failed and have thus far only been successful for phages of smaller genome size [26]. The engineering of such large bacteriophages therefore requires homologous recombination-based approaches, which in turn necessitate the delivery of a suitable homologous recombination template into a phage-sensitive host cell [9]. *S. aureus* poses inherent difficulties for the introduction of foreign DNA due to the presence of numerous potent intracellular restriction-modification (R-M) systems which are characteristic for the species [27, 28]. To this end, the engineering of *S. aureus* targeting, lytic bacteriophages with broad host range has proved difficult thus far [29].

Reporter-phages are engineered phages which result in a detectable signal upon infection of a sensitive bacterial host. The inherent high host specificity of bacteriophages makes them an ideal alternative for rapid bacterial diagnostics. Methods for bacterial detection find widespread usage in food preservation and production as well as animal and human health. These are often time consuming and can be limited in the range of detectable pathogens, especially those which are less common [30, 31]. The inherent high host specificity of phages makes them an ideal alternative for rapid bacterial diagnostics. The emission of a signal when a bacteriophage infects a host strain is typically achieved

through integration of a bioluminescent protein-coding payload gene into the phage backbone [23, 32]. These payloads are usually less problematic to engineer compared to e.g. genes coding for antimicrobial compounds, as they have little to no toxic effect on the host and therefore tend to have no impact on infection kinetics and production of viable phage progeny.

Here, we report on a genetically engineered phage K, K::*nluc*, capable of infecting a wide range of clinical *S. aureus* isolates with varying degrees of vancomycin resistance. The introduction of a nanoluciferase (*nluc*) payload allows for a rapid and discriminate detection of *S. aureus* strains. Finally, with the development and optimization of an assay for the detection of *S. aureus* in human whole-blood, we show the functionality and efficacy of K::*nluc* even in complex matrices such as bovine raw milk and human whole blood, where potential interactions with components such as immune factors often inhibit *in vivo* bacteriophage infection.

Results

Homologous recombination-based, CRISPR-Cas9-assisted engineering of *Staphylococcus* reporter phage K::*nluc*

K is a *Staphylococcus*-specific bacteriophage with a broad host range extending across multiple species of *Staphylococci* [33]. The use of K as an engineering backbone therefore ensures a wide range of detection for our intended reporter phage system. Furthermore, the large (∼150 kb) genome lowers the chances of the payload being too large for integration due to its relatively small size compared to the rest of the genome. We elected to use an *Oplophorus gracilirostris*-derived, bioluminescent luciferase-coding gene (NanoLuc®, Promega) which is merely 516 bp in length and has previously been reported to produce a detectable signal even at very low concentrations, compared to other analyzed reporter payloads [23, 34-38]. Although engineering of an *S. aureus* infecting lytic bacteriophage has, to our knowledge, not previously been reported, such attempts for other Gram-positive pathogens [11-14] motivated us to attempt to use a CRISPR-Cas9-assisted, homologous recombination-driven approach to integrate the *nluc* reporter gene as a payload. Transformation of *S. aureus* with foreign DNA is non-trivial [27, 28]. We therefore elected to use the restriction-modification deficient laboratory strain RN4220 (DSM 26309) [39, 40] which has much higher transformation yields and allowed us to establish a two-step protocol as shown in **Figure 1A**. First, phage K is propagated in the presence of the homology donor plasmid (pEDIT), which contains the *nluc* gene, as well as up- and downstream homology arms directing sequence-specific integration of the payload behind the major capsid protein (CPS)-coding gene on the phage backbone. We selected an integration site downstream of *cps* as it typically has high expression levels and has frequently been used as a payload insertion site in the past [41, 42]. The resulting phage lysate contains a mixed population of very few recombinant phages and a vast majority of wildtype phages. This lysate was subsequently propagated on a second

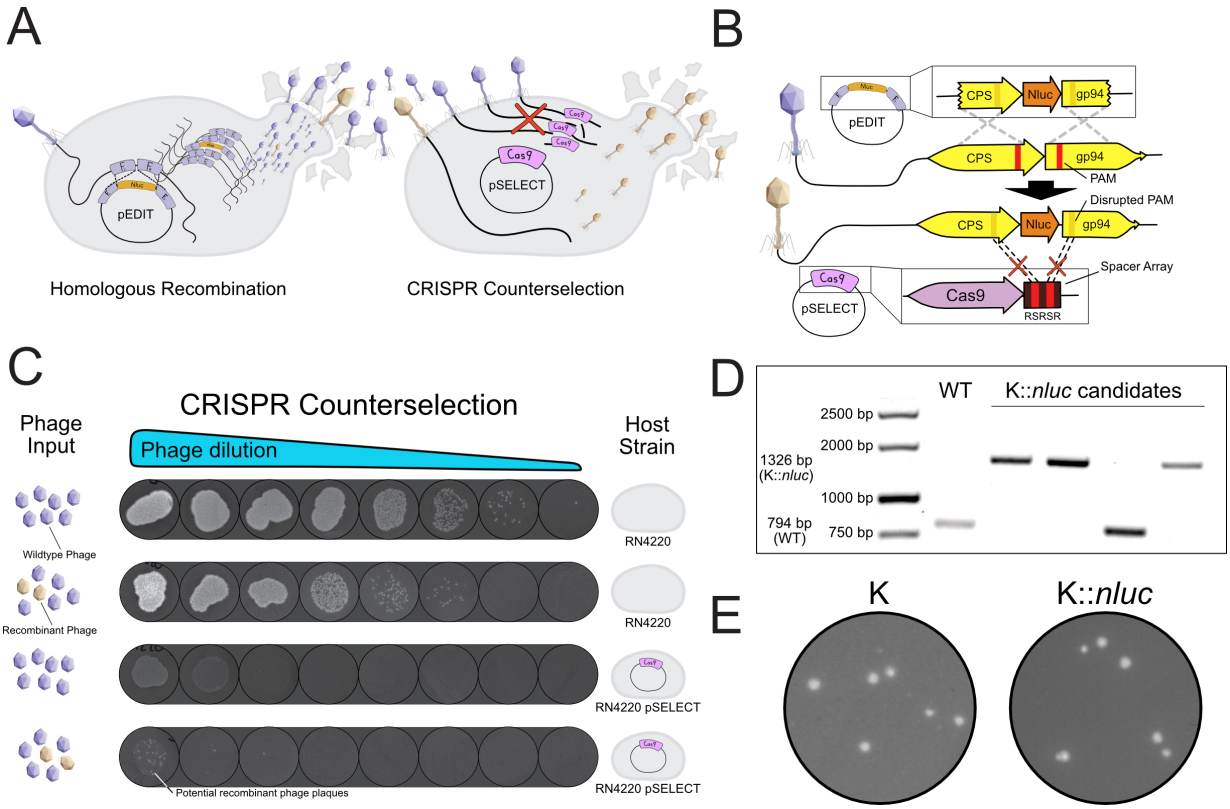


Figure 1: Construction of reporter phage K::*nluc* using a homologous recombination-based and CRISPR-Cas9-assisted phage engineering platform.

(A) Two *S. aureus* bacterial hosts function as donor strain and counterselection strain through integration of pEDIT and pSELECT plasmids, respectively. Through sequential infection of the two hosts via soft-agar overlay, plaques potentially containing recombinant phage can be isolated, purified and sequenced to confirm the intended genotype. **(B)** The complementary design of pEDIT and pSELECT allows for the exclusive selection of phage particles whose packaged DNA has undergone homologous recombination. This is facilitated through two synonymous, single-nucleotide polymorphisms in the CRISPR-Cas9 targeting protospacer adjacent motifs (PAM) in the donor template. Cas9 restriction of phage DNA which has not undergone homologous recombination is facilitated through the rational design of PAM-flanking guide RNAs on pSELECT. The efficiency of the CRISPR-Cas9 counterselection system is shown in **(C)**. The top two rows show spot-on-lawn infection assays of wildtype lysate and lysate having undergone homologous recombination on the wildtype bacterial host RN4220 with similar results between the two. Row 3 shows fully inhibition of plaque formation for wildtype K when the bacterial host contains pSELECT. The final row contains several visible plaques at the lowest dilutions, indicating the presence of phage variants which have most likely escaped CRISPR-Cas9 restriction. Individual plaques were picked and presence of an insertion of the intended size was validated using PCR, results of which can be seen in **(D)**. Positive PCR products were Sanger sequenced to determine the correct genotype at the insertion site. **(D)** Plaque morphologies of K and K::*nluc*.

host-strain containing the counterselection plasmid (pSELECT), which encodes an episomal CRISPR-Cas system designed to restrict wildtype phage genomes exclusively, while keeping recombinant genomes intact and allowing for their enrichment. We designed pSELECT to contain the *S. pyogenes* Cas9 gene, tracrRNA and a crRNA element with two spacers which respectively target two CRISPR-Cas9-targeted protospacer adjacent motifs (PAM) proximal to the *nluc* integration site, with the corresponding engineering template designed to lack both PAMs in K::*nluc* (see **Figure 1B**). Our counterselection system showed 100% restriction of wildtype phage and allowed for easy and rapid identification of recombinant escape mutants, with the majority of mutants containing the intended payload insertion (**Figure 1C and D**). There was no evident different in plaque morphology between the wildtype and engineered phages (**Figure 1E**).

Characterization of K::*nluc* infectivity and bioluminescence kinetics across clinically relevant *S. aureus* strains

To exclude potential loss of infectivity or host range due to the genetic alteration, we evaluated the efficiency of plating (EOP) of K::*nluc* on a selection of 71 *S. aureus* strains (**Suppl. Figure S1**) consisting of a variety of different strains, including the phage propagation host PSK, common laboratory strains and clinical isolates with varying degrees of vancomycin resistance. We experimentally determined the minimum inhibitory concentration (MIC) to vancomycin for all strains as described in [43] and Materials and Methods (**Suppl. Figure S2**). One vancomycin susceptible (VSSA), one resistant (VRSA), and one intermediate resistant (VISA) *S. aureus* strain showing high efficiency of plaquing (EOP) when infected with K::*nluc*, was representatively selected to determine the kinetics of bioluminescence emission over time upon infection with K::*nluc*. All three reactions (hosts: PSK, LI6, VRSA7) showed a similarly rapid and steady rise of bioluminescence until plateauing at a peak fold-change of $\sim 1 * 10^6$ relative light units (RLU) above background luminescence after about 3 hours (**Figure 2A**). To further evaluate

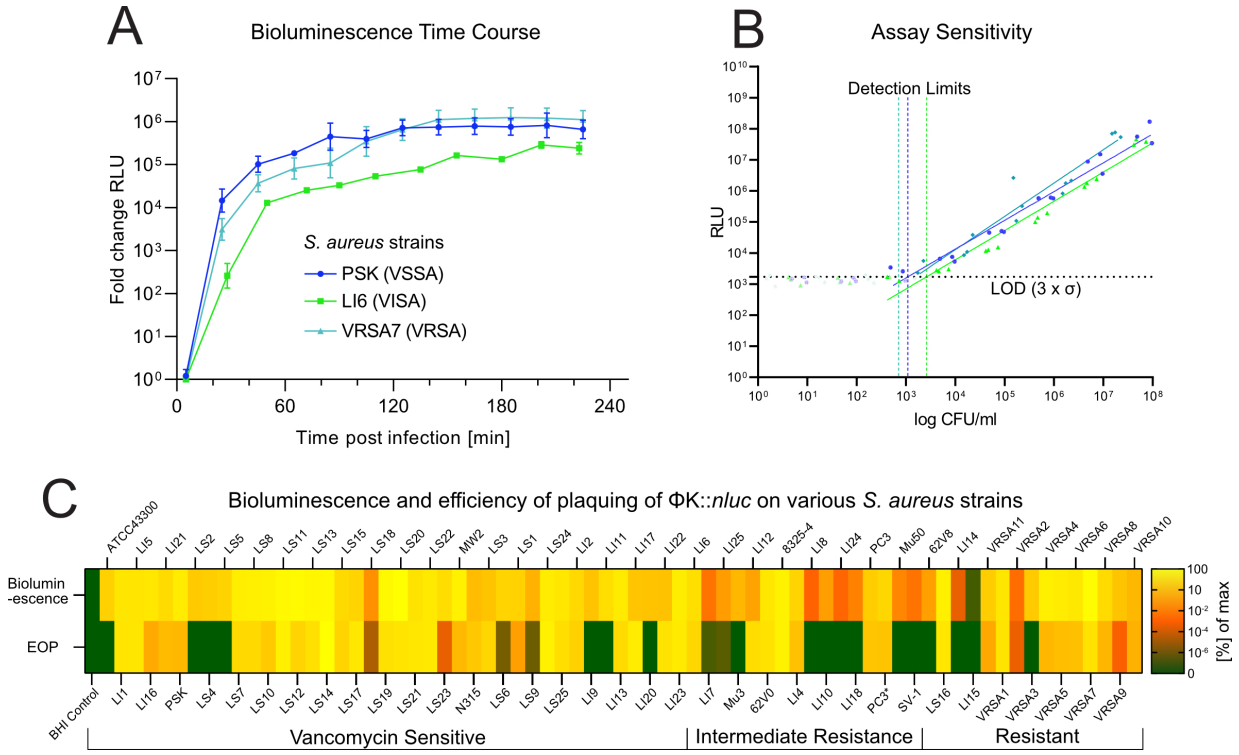


Figure 2: Phage K::*nluc* enables rapid, sensitive, and broad host-range detection of *S. aureus* in *in vitro* assays.

(A) Bioluminescence time course measurements for *S. aureus* strains PSK, LI6 and VRSA7 were obtained by calculating the fold change in relative light units (RLU) compared to infection with K wildtype at a bacterial density of OD600=0.01 and phage titer of $5 * 10^7$ PFU/ml. Values are corrected for background luminescence. Associated error bars represent mean +/- standard deviation (n=3). **(B)** Minimal dose response of LI6, PSK and VRSA7 to K::*nluc* was determined by measuring the RLU after 3h of infection for varying bacterial concentrations. Values below the determined limit of detection, set at 3 standard deviations of the mean background luminescence, were excluded. The detection limit (vertical dotted lines) were calculated as the minimum cell number required to produce a reliable signal (three standard deviations (σ)) above the background luminescence (horizontal dotted line). **(C)** Bioluminescence and efficiency of plaquing (EOP) for 71 *S. aureus* isolates. Bioluminescence for each strain is represented as the mean measured bioluminescence 3h post infection (n=3). Efficiency of plating is given by the number of plaque forming units (PFU) after infection of a specific strain with K::*nluc*. Values are relative to the host with the highest measurement (LS20 for bioluminescence, LS14 for EOP).

the sensitivity of our reporter phage system, we determined the minimum dose response of PSK, LI6 and VRSA7-emitted luminescence upon infection with a fixed concentration of K::*nluc* (**Figure 2B**). Although the minimal dose response of LI6 (2617 CFU/ml) was slightly higher than that of PSK (1093 CFU/ml) and VRSA7 (707 CFU/ml), all remained within comparable ranges of previously established reporter phage systems [34, 37]. We next compared the K::*nluc* produced bioluminescence across all 71 *S. aureus* strains and compared it with the previously determined EOP (**Figure 2C**). Bioluminescence was detectable across all species, including those which previously showed no evident plaque formation.

K::*nluc* detection of vancomycin susceptible (VSSA), resistant (VRSA), and intermediate resistant (VISA) *S. aureus* in human whole blood and bovine raw milk.

Although bacteremia constitutes the vast majority of reported bloodstream infections [44, 45], blood constitutes a much more complex matrix than standard laboratory culture conditions and previously observed *in vitro* infection kinetics between K::*nluc* and the pathogenic bacterial strains is not guaranteed in such a setting [46-48]. Furthermore, next to direct effects resulting in bacteriophage inactivation, the presence of a multitude of immune factors may likely have an influence on bacterial metabolism, growth and consequently phage infection kinetics. We therefore elected to evaluate the functionality of our reporter phage system in whole human blood. Assay conditions were optimized using whole human blood spiked with strain PSK as shown in **Figure S3**. The fastest and strongest signal response was obtained when using the following conditions: Spiked blood was diluted five-fold in growth medium and the cells incubated for 1 h at 37°C to activate host cell metabolism prior to reporter phage infection. We found the signals to be higher when using AC1 (Na3-citrate, citric acid, glucose, potassium sorbate) as an anticoagulant rather than AC2 (Li-Heparin). Using these optimized conditions, we

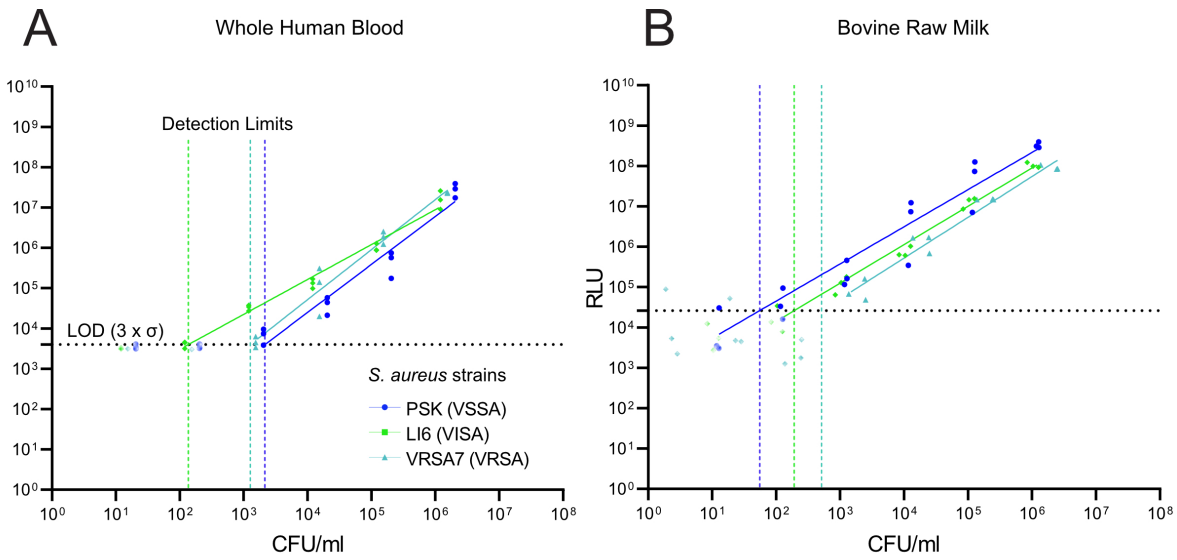


Figure 3: Detection of vancomycin resistant *S. aureus* in whole human blood and bovine raw milk.

Minimal dose response of LI6, PSK and VRSA7 to K::*nluc* in whole human blood **(A)** and bovine raw milk **(B)** was determined by measuring the RLU after 3h of infection for varying bacterial concentrations. Values below the determined limit of detection, set at 3 standard deviations of the mean background luminescence, were excluded. The detection limits (vertical dotted lines) were calculated as the minimum cell number required to produce a reliable signal (three standard deviations (σ)) above the background luminescence (horizontal dotted line).

determined the detection limits using the previously selected *S. aureus* strains PSK, LI6 and VRSA7 (**Figure 3A**). PSK, LI6 and VRSA7 could be detected at concentrations as low as 2151, 136 and 1270 CFU/ml, respectively, which presents only a marginal increase compared to the detection limits observed in BHI for PSK and VRSA7, and even lower detection limits than previously observed for LI6. This indicates little to no effect of the blood constitution on infection kinetics using this setup. Bovine raw milk constitutes a further complex matrix reported to have negative effects on bacteriophage proliferation on host bacteria [49]. Previous studies analyzing the infectivity of phage K in bovine milk show inhibition of bacterial lysis by the phage due to the strong absorption of Whey proteins present in the milk to the bacterial cell surface [50, 51]. To this end, we elected to repeat the previous experiment while exchanging blood for unpasteurized, raw bovine

milk. Against expectations, treatment of bovine raw milk infected with *S. aureus* strains PSK, LI6 and VRSA7 with K::*nluc* yielded comparable levels of bioluminescence and minimal dose responses of 55, 191 and 514 CFU/ml, respectively, showing that with our optimized setup, reliable detection of PSK by K::*nluc* in bovine raw milk is possible.

Discussion

The rising number of antimicrobial resistant pathogens in recent decades has rekindled the interest in using bacteriophages to combat infectious diseases [52-54]. Paralleled by milestone advances in genetic engineering and synthetic biology, engineered phage variants have become of particular interest in the context of patient-tailored treatments and precision medicine [7-9]. Here, we report on the development of a genetically engineered *Staphylococcus aureus*-infecting lytic bacteriophage containing a nanoluciferase bioluminescent reporter payload, K::*nluc*. We demonstrate K::*nluc* efficacy for reliably detecting a wide range of antibiotic resistant clinical *S. aureus* isolates, analyze the *in vitro* infection kinetics and quantify minimal dose response in regular growth media as well as complex matrices such as human whole blood and bovine raw milk.

The methodology presented here is based on homologous recombination-based bacteriophage engineering, followed by a subsequent CRISPR-Cas9-assisted counterselection (**Figure 1A**) as previously reported for other pathogenic bacterium/bacteriophage combinations [55-57]. The delivery of exogenous DNA into *S. aureus* cells is typically non-trivial, largely due to diverse intracellular defense mechanisms which lead to exceedingly low transformation efficiency in most *S. aureus* strains [28]. The ability of K to infect the widely used laboratory strain RN4220 allowed us to introduce the homologous-recombination and counterselection plasmids needed for engineering the phage.

We selected phage K as the engineering "backbone" based on multiple factors. K has been shown to generally have a large host range, primarily in *S. aureus*, but also shows a complex interaction stoichiometry across various *Staphylococcal* species [33]. Although this presents an advantage in regards to the total amount of strains we can detect using K::*nluc*, *S. aureus* infection frequently occurs in the presence of other bacteria, such as the commensal *Staphylococcus epidermidis* [58], which may lead to false positives. Nevertheless, the naturally broad host range of K elevated the chances of our reporter

phage to be able to detect a large number of the selected, clinically relevant *S. aureus* strains.

Reporter phages are phages engineered to contain a reporter protein payload, usually in the form of a fluorescent/luminescent protein. This allows the detection of pathogenic bacteria in applications such as food safety and clinical diagnostics. Compared to antimicrobially active payloads, which are inherently invasive on the bacterium/phage interaction kinetics and thereby often incur a fitness cost on the engineered phage, reporter proteins are not likely to have any significant effect on bacterial metabolism and thus not disrupt phage infection. The bioluminescent nanoluciferase NanoLuc® has previously been successfully used for reporter phage development [35-37, 59]. We elected to test our engineering workflow by engineering a reporter gene-coding phage K, K::*nluc*.

Antimicrobial resistant variants such as vancomycin resistant (VRSA) and methicillin resistant (MRSA) *Staphylococcus aureus* are widespread with a regular emergence of new resistant strains [60-62]. In order to evaluate the efficacy of K::*nluc* in a clinical context, we acquired a diverse selection of 71 *S. aureus* strains, including clinical isolates from infected patients, which had been determined to have varying degrees of vancomycin resistance (**Suppl. Figure 2**). Although several bacterial strains showed no active infection of K::*nluc* in standard soft-agar overlay infection screens, bioluminescence could nevertheless be detected for all strains. This implies that both binding and DNA delivery of K::*nluc* occurs. However, in cases where plaque formation is not evident, the infection cycle is likely disrupted before lysis and release of progeny phage particles can take place. This has significant positive implications for application in a clinical setting, as the determined range of detection of K::*nluc* using bioluminescence exceeds that of detection using conventional plaque assays (**Figure 2C**). This allows for detection of pathogenic *S. aureus* even when progressive productive infection of the phage in the bacteria is not guaranteed. Furthermore, the ability of K::*nluc* to detect bioluminescence across the full selection of 71 strains implicates a broad-coverage of further circulating

S. aureus strains.

S. aureus can cause a number of diseases and poses a significant global public health burden. The successful development and application of a K::*nluc*-based *S. aureus* screening assays optimized for whole human blood and bovine raw milk (**Figure 3**) demonstrate a potential clinical and veterinary application, respectively. Both human whole blood and bovine raw milk constitute complex matrices with immune factors and other compounds potentially inhibitory for bacteriophage/host interaction. Indeed, it has been shown previously for phage K specifically that the presence of Whey proteins in bovine raw milk competitively inhibit cell surface attachment of the phage, thereby significantly inhibiting infection efficiency [50, 51]. In contrast, no significant reduction of K::*nluc*-expressed bioluminescence could be observed in our experiments. It is very likely that the effect of competitive inhibition of Whey protein was mitigated due to the dilution with growth medium prior to inoculation with K::*nluc* in our setup. An alternative explanation is a possibly low baseline concentration of Whey protein in our milk sample, since significant deviations in Whey protein concentrations of milk from different cows could be observed in [50].

Although engineered bacteriophages such as K::*nluc* are generally considered promising alternatives to current treatment regimes, several caveats exist which impede the immediate implementation of such phage-based treatment and diagnostics for *S. aureus* infections. For example, although *S. aureus* is often the causative agent of bacteremia and resulting sepsis, onset of severe symptoms is rapid and clinical intervention, usually in the form of broad-spectrum antibiotics, must proceed immediately without prior determination of the causative pathogen. Nevertheless, the increasing shift towards patient-tailored treatments and precision medicine shows promise for the application of such an *S. aureus* reporter phage, for instance as a companion diagnostic tool for *S. aureus*-directed phage therapy or for chronic (e.g. wound) infections.

In conclusion, the establishment of a functional engineering pipeline for *S. aureus*-

targeting bacteriophages is the first of its kind and shows great promise for future diagnostic and potentially even treatment regimes, specifically in the context of MDR *S. aureus* in a clinical setting.

Materials and Methods

Vancomycin minimum inhibitory concentration (MIC) of selected *S. aureus* strains

The minimum inhibitory concentration (MIC) of the 71 *S. aureus* strains used in this study was determined using the standard culture conditions described in [33]. Briefly, 96 well plates were prepared, with each well containing 250 μl Miller-Hinton broth supplemented with various vancomycin concentrations (range 0.0625 μg/ml – 64 μg/ml with twofold increase between subsequent concentrations). Wells were inoculated with 2 μl of 1:1000 diluted bacterial cultures (grown overnight, 37°C) for each of the 71 strains in triplicate. Plates were incubated for 18h at 37°C and turbidity measured photometrically (OD_{600}). Wells with an $OD_{600} > 0.1$ were deemed turbid.

Bacterial strains and culture conditions

S. aureus PSK (ATCC 19685) was used as propagation and *S. aureus* RN4220 (DSM 26309) as the engineering host of K and K::*nluc*. *E. coli* XL1-blue MRF' (Stratagene) was used for plasmid amplification prior to RN4220 transformation. RN4220 and XL1-blue cultures were grown over night (O/N) at 37°C in in Brain Heart Infusion (BHI) Broth (Biolife Italiana) and Luria-Bertani/Lysogeny broth (LB) medium (3M sodium chloride, 10 g/L tryptone, 5 g/L yeast extract, pH 7.2), respectively. The selection of 71 laboratory strains and clinical isolates (**Figure S2**) were grown on BHI Broth with corresponding antibiotic supplements.

Bacteriophage propagation

Phage K was propagated on *S. aureus* PSK using the soft-agar overlay method with BHI as bottom agar and LC agar (LB supplemented with 10 mM CaCl2, 10 mM MgSO4, 10g/L glucose) as top agar. Overlays were incubated O/N at 37 °C and phages extracted

from plates with semi-confluent lysis using 5 mL SM buffer (4 °C, 2 h, constant agitation). Lysates were sterile-filtered (0.22 μm pores). Phage particles were precipitated O/N at 4°C using polyethylene-glycol (7% PEG8000 and 1 M NaCl) and purified using stepped CsCl gradient ultracentrifugation. The obtained phage suspension was dialyzed twice against 1000x excess of SM buffer. Purified samples were then stored long-term at 4°C. The titer was determined using the soft-agar overlay method.

Electroporation of bacterial strains

XL1-blue electrocompetent cells were electroporated at 2.5 kV, 200 Ω, 25 μF, incubated for 1 h with SOC recovery medium (2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 20 mM Glucose) at 37 °C, and plated on selective agar to isolate successful transformants. RN4220 electrocompetent cells were electroporated at 1.8 kV, 600 Ω, 10 μF with 500-1000ng of amplified plasmid DNA, incubated for 1 h at 37°C in B2 recovery medium (10 g/l casein hydrolysate, 5 g/l D-glucose, 1 g/l potassium phosphate dibasic, 25 g/l NaCl, 25 g/l yeast extract) and plated on selective medium to isolate successful transformants.

Plasmid design

The plasmid pLEB579 (kindly gifted by T. Takala, University of Helsinki, Finland) is a shuttle vector shown to have high transformation efficiencies in both *E. coli* and *S. aureus* and was therefore used as a backbone for both the editing template (pEDIT) and the CRISPR-Cas9-counter-selection system (pSELECT). We used a previously reported, *Streptococcus pyogenes*-derived Cas9 (SpyCas9)-based CRISPR system [58] and exchanged the two spacers in the repeat-spacer-repeat-spacer-repeat (RSRSR) region. This was done to allow targeted restriction of wildtype phage K at two distinct loci (8304 bp and 148 bp up- and downstream of the intended insertion site, respectively) designed to contain a PAM-disrupting synonymous mutation in the engineered phage

variants. pEDIT was constructed by integrating an *nluc* gene (optimized for *S. aureus* codon usage, avoidance of Rho-independent termination and an added upstream ribosome binding site: GAGGAGGTAAATATAT), flanked by 400 bp (upstream) and 300 bp (downstream) homology arms, corresponding to the intended K insertion site, into the linearized pLEB579 backbone. Two silent point mutations were included to disrupt the two PAMs targeted by the CRISPR spacers (**Figure 1B**). All synthetic sequences were acquired as GeneArt String DNA Fragments (Thermo Fischer), albeit the RSRSR sequences, which were ordered as GeneArt Gene Synthesis (Thermo Fischer) (**Table S2**). Assembly of all constructs was performed using isothermal GibsonAssembly® reaction (NEBuilder® HiFi DNA Assembly Master Mix) and subsequently transformed into *E. coli* XL1-blue MRF' for plasmid amplification. Plasmids and primers are listed in (**Table S1**).

CRISPR-Cas9-assisted engineering of K::*nluc*

pEDIT and pSELECT were transformed into *S. aureus* RN4220 to acquire the strains required for recombination and counter-selection, respectively. RN4220 pEDIT was infected with K via soft-agar overlay and a high titer lysate obtained as described previously (see *bacteriophage propagation*). Plates showing semi-confluent lysis were washed with SM buffer and 10-fold dilutions of the resulting lysate were used to perform soft-agar overlays on RN4220 pSELECT. Individual plaques were picked from the plates showing the fewest (non-zero) plaques, resuspended in SM buffer, and clonally isolated by three rounds of plaque-purification. PCR amplification using primers flanking the insert site (**Table S1**) was performed and products showing a size indicative of the intended insertion purified and Sanger sequenced (Microsynth AG, Balgach, Switzerland) to validate the correct genomic sequence. Validated phage lysates were purified using ultracentrifugation on a caesium-chloride gradient and subsequent dialysis as described in the section *bacteriophage propagation*.

Soft-agar overlay

5 ml BHI soft-agar were melted and cooled to 47°C. The molten soft-agar was inoculated with 200 μl bacterial culture of adequate turbidity ($OD_{600} > 1$) and 10 μl of phage suspension, briefly mixed by agitation, and spread evenly onto BHI agar plates with. Plates were let dry for 15 minutes at room temperature (RT) and subsequently inverted and incubated at 37°C for 12-18 h.

Spot-on-lawn assay

5 ml BHI soft-agar were melted and let cooled to 47°C. The molten soft-agar was inoculated with 200 μl of bacterial culture, briefly mixed my vortexing, and spread evenly on BHI agar plates. Plates were dried at RT for 15 minutes. 10 μl droplets of phage suspension were then placed carefully on the dried soft-agar. Plates were dried for 15 min, inverted, and incubated at 37°C for 16 h.

Determination of efficiency of plaquing (EOP)

EOP of bacteriophage suspensions was determined by performing spot-on-lawn assays on bacterial strains of interest. 10-fold dilutions of the phage suspension were prepared up to a maximum dilution of $1 * 10^{-8}$. Spot-on-lawn assays were performed as described in the previous section using the series of bacteriophage dilutions and O/N cultures of the corresponding host strains .

Bioluminescence time course assay

Stationary phase bacterial cultures were diluted to OD_{600} 0.01, inoculated with $5 * 10^7$ PFU/ml K::*nluc* and incubated at 37°C (180 rpm agitation). Bioluminescence measurements were taken by combining 25 μl of the sample solution with an equal volume of prepared buffer-reconstituted *nluc* substrate as detailed by the manufacturer (Nano-Glo Luciferase Assay System; Promega). Measurements were taken every 20 min (225

min total) in Nunc™ F96 MicroWell™ 446 plates using a GloMax® navigator luminometer (Promega) with 5 s integration time and 2 s delay. To determine the background-corrected fold-change in relative light units (RLU), measurements were normalized to a control reaction of K::*nluc* in BHI medium and the fold-change was calculated as the difference in RLU to an infection of the same strain with wildtype K.

Minimal dose-response

To determine the minimum concentration of cells giving a significant bioluminescence signal above the background, stationary phase bacterial cultures were diluted to a range of concentrations in ten-fold increments (OD_{600} $10^{-1} - 10^{-10}$). Measurements were taken 3 h post-infection ($5 * 10^7$ PFU/ml) and background corrected as described in the previous section. The lowest detectable concentration of cells was determined as the lowest measurement above a signal threshold 3 times the standard deviation of the background signal. The theoretical minimal concentration was determined as the intersection of the determined signal threshold and a linear regression of the data with RLU values greater than the threshold.

Determination of bioluminescence for the 71 *S. aureus* strains used in the study.

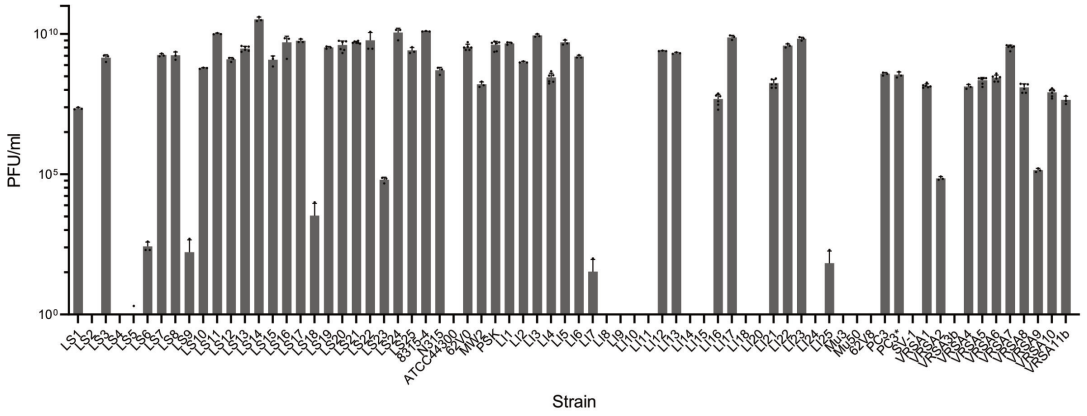
Stationary phase bacterial cultures were diluted to OD_{600} 0.01, inoculated with $5 * 10^7$ PFU/ml K::*nluc* and incubated at 37°C (180 rpm agitation). Fold-change bioluminescence was measured at 3 hours post-infection and background corrected.

K::*nluc*-based detection of *S. aureus* in patient blood and bovine raw milk

Minimal dose response of K::*nluc* infection on one representative each of VSSA, VISA and VRSA was conducted in triplicate as done with regular growth medium described above,

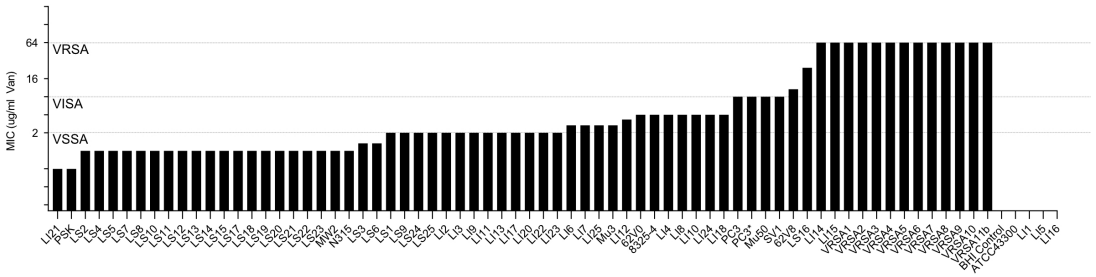
albeit with some modifications. First, spiked whole human blood or bovine raw milk were mixed 1:5 with BHI growth medium and incubated 1h at 37°C with agitation (180 rpm) prior to infection with $5 * 10^7$ PFU/ml K::*nluc*. Bioluminescence was measured after 3 h. The whole human blood samples were stored in anticoagulant solutions containing either 1.89 mg/ml Na3-citrate, 0.69 mg/ml citric acid, 2.1 mg/ml glucose and 0.03 mg/ml potassium sorbate (BD Vacutainer® (REF 367756), Becton, Dickinson and Company, New Jersey, USA) or Li-Heparin (17 IU/mL) (BD Vacutainer® (REF 368886, Becton, Dickinson and Company, New Jersey, USA).

Supplementary Material



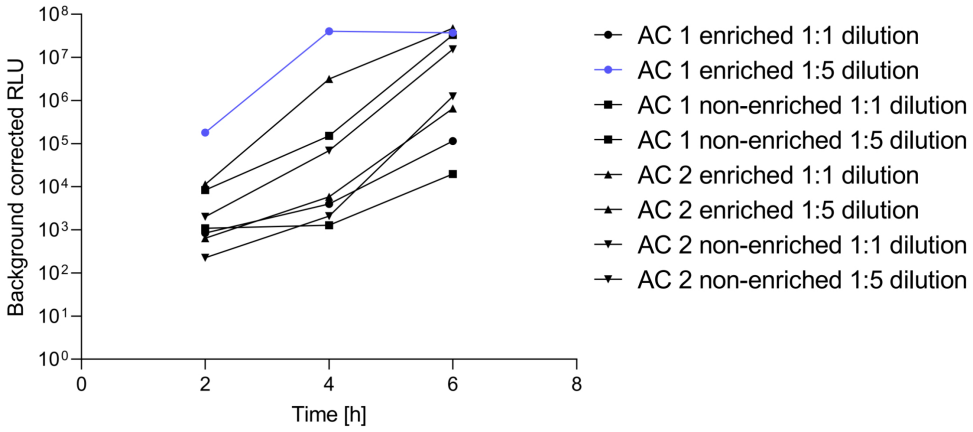
Supplementary Figure 1: Efficiency of plating (EOP) of K::*nluc* on a selection of 71 *S. aureus* species

Standard spot-on-lawn plaque assay was performed to elucidate the absolute EOP of K::*nluc* on a panel of 71 *S. aureus* species given by plaque forming units per volume (PFU/ml). Standard deviation was calculated from 3-6 replicates per strain.



Supplementary Figure 2: Minimal inhibitory concentration (MIC) of vancomycin on bacterial strains used in this study

The minimum inhibitory concentration (MIC) of vancomycin on the 71 bacterial strain used in this study was tested by culturing in the presence of varying vancomycin concentrations, ranging from 0.0625 μg/ml – 64 μg/ml with twofold increase between subsequent concentrations. For cases where replicates did not yield uniform results, the MIC was determined as the triplicate mean. Vancomycin suceptibility (VSSA), intermediate resistance (VISA) and full resistance (VRSA) are indicated along with the MIC cutoffs defined by [43].



Supplementary Figure 3: Effects of various parameters on K::*nluc* infection-associated bioluminescence in human whole blood.

Whole human blood was spiked with different bacterial concentrations of *S. aureus* PSK. Samples were subsequently diluted with BHI growth medium at a ratio of 1:1 or 1:5. Samples were then either infected with $5 * 10^7$ PFU/ml K::*nluc* or first incubated at 37°C for 1 h prior to infection with K::*nluc*. These parameters were furthermore tested on two types of anticoagulant solutions, AC1 (Na3-citrate, citric acid, glucose, potassium sorbate) and AC2 (Li-Heparin). The combination of parameters resulting in the highest bioluminescence is highlighted in blue.

Supplementary Table 1: Primers and templates used for plasmid construction and insert confirmations.

Fragment	Template	Primer 1	P1 Sequence [5' -> 3']	Primer 2	P2 Sequence [5' -> 3']	Amplicon Size (bp)
pLEB579 SpyCas9 Backbone	pLEB579 SpyCas9 T4*	pLEB579 SpyCas9 Backbone Fw	GACTCCATTCAACATTGCCGA	pLEB579 SpyCas9 Backbone Rev	TCAGCTAGACTTCAGTCTTG AAAAG	7903
pLEB579 SpyCas9 K Spacers	Phage K Spacers Synthetic DNA String	Phage K Spacers Fw	GCAGTAATACAGGGGCTTTT C	Phage K Spacers Rev	CCTCTTTCTCAAGTTATCATC GG	350
pLEB579 Backbone	pLEB579	pLEB579 Backbone Fw	AGTCGATGTTAAACCGTGTG CTCTACG	pLEB579 Backbone Rev	CGCGCTATTAATCGCAACAT CAAACC	2848
pLEB579 ϕK_nluc_CPS Insert	Phage K Nanoluc Post Capsid Synthetic DNA String	Phage K Nluc HR Insert Fw	GGGGCTTTTATTTTGGTTTGA TGTTG	Phage K Nluc HR Insert Rev	TTATAGTTTTGGTCGTAGAG CACACG	1312
pEDITϕK_nluc _CPS Confirmation	pEDITϕK_nluc_C PS	pEDIT Confirmation Fw	GAGAAATGGAAGTTGAATTA AG	pEDIT Confirmatio n Rev	GATAATGAACTGTGCTGATT AC	1616
Phage K::nluc Confirmation	Phage K::nluc	Phage K::nluc Confirmation Fw	AGTAGTAGTTAACTCAGATG AC	Phage K::nluc Confirmatio n Rev	TGTAGATTTTCTAGTAGTAT TTGTAG	1326

* From [33].

Supplementary Table 2: SpyCas9 Phage K spacer string and pEDIT Phage K::*nluc* string used in this study.

Synthetic String Name	Sequence [5' -> 3']
SpyCas9 Phage K Spacer String	AGTATATTTTAGATGAAGATTATTTCTTAATAACTAAAAATATGGTATAATACTCTTAATAAATGCAGTAAT ACAGGGGCTTTTCAAGACTGAAGTCTAGCTGAGACAAATAGTGCGATTACGAAATTTTTTAGACAAAAATA GTCTACGAGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACAGGAAGTACATACCTGTTTCTTTACCT TGAGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACATAAAAAAATGGCTACTGTTTATGGTACAGGT TTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACTTCAGCACACTGAGACTTGTTGAGTTCCATGTTTTAGA GCTATGCTGTTTTGAATGGACTCCATTCAACATTGCCGATGATAACTTGAGAAAGAGGGTTAATACCAGCA GTCGGATACCTTCCTATTCTTTCTGTTAAAGCGTTTTCATGTTATAATAGGCAAAAGAAGAGTAGTGTGATC GTCCATTCCGACAGCATCGCCAGTCACTAT
pEDIT Phage K Nanoluc String	AAAGTCGAAGGGGGCTTTTATTTTGGTTTGATGTTGCGATTAATAGCGCGAGTAACAGCTACAGTATCTAA CGTAGACGATGGTGTTAAACTTTCAATTAATGTTAACGCTATGTACCAACAACAACCACAATTCGTTTCTAT CTATCGTCAAGGTAAAGAAACAGGTATGTACTTCCTAATCAAACGTGTACCAGTTAAAGATGCACAAGAA GACGGAACAATCGTATTCGTAGATAAGAACGAAACATTGCCTGAAACAGCAGACGTATTTGTTGGTGAAA TGTCACCACAAGTAGTTCACTTATTCGAATTACTTCCAATGATGAAATTACCATTAGCTCAAATTAATGCTTC TATTACATTTGCAGTATTATGGTATGGTGCATTAGCATTACGTGCTCCTAAAAAATGGGCTCGTATTAAAAA CGTTCGTTATATCGCAGTTTAAGAGGAGGTAAATATATATGGTATTCACTTTAGAAGATTTCGTAGGTGAT TGGCGTCAAACTGCTGGTTACAACTTAGATCAAGTATTAGAACAAGGTGGTGTATCATCATTATTCCAAAA CTTAGGTGTATCAGTAACTCCAATCCAACGTATCGTATTATCAGGTGAAAACGGTTTAAAAATCGATATCC ACGTAATCATCCCATACGAAGGTTTATCAGGTGATCAAATGGGTCAAATCGAAAAAATCTTCAAAGTAGTA TACCCAGTAGATGATCACCACTTCAAAGTAATCTTACACTACGGTACTTTAGTAATCGATGGTGTAACTCCA AACATGATCGATTACTTCGGTCGTCCATACGAAGGTATCGCTGTATTCGATGGTAAAAAAATCACTGTAAC TGGTACTTTATGGAACGGTAACAAAATCATCGATGAACGTTTAATCAACCCAGATGGTTCATTATTATTCCG TGTAACTATCAACGGTGTAACTGGTTGGCGTTTATGTGAACGTATCTTAGCTTAATAGAATAAGAAAAACT GAATACAAGAGAATAGGGATAAACTTAGGGTTTATCCCTTTTTTATTAAAATAAACTTGAAGGGATTTAAT AAATATGTTATACTATAAGAAACTATTAGATAAAAAAATGGCTACTGTTTATGGTACAGTTGAGATTGACA AAGATGGAGTAGTCAAAGGATTAACTAAAGAACAAGAAAAAGAATTTGCCAATGTTCCAGGTTTTGAATT TGAAGAAGAAAAGAAAACTACTAGAAAACAATCAGCTTCTACTAGTAAAGAAGAAGAGCCTAAGGAAGA GGAAGTCGATGTTAAACCGTGTGCTCTACGACCAAAACTATAAAACCTTTAAG

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2.3 Manuscript III: Genetic engineering and rebooting of bacteriophages in L-form bacteria

Specific contribution by the doctoral candidate

The bacterial strains Rev2L and SuL27 were generated and provided by co-authors SK and SM, respectively. All further laboratory experimentation, including generation of bacterial strain LeFred, was conducted by the doctoral candidate. The manuscript was written in full- and first-authored by the doctoral candidate. Figures were designed and created by the doctoral candidate. The manuscript was reviewed and revised by SK and SM.

Genetic engineering and rebooting of bacteriophages in L-form bacteria

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Running head: Bacteriophage engineering and rebooting in L-form bacteria

Abstract

The rapid increase of circulating, antibiotic-resistant pathogens is a major ongoing global health crisis and arguably, the end of the "golden age of antibiotics" is looming. This has led to a surge in research and development of alternative antimicrobials, including bacteriophages, to treat such infections (phage therapy). Isolating natural phage variants for the treatment of individual patients is an arduous and time-consuming task. Furthermore, employing natural phages is frequently hampered by natural limitations, such as moderate in vivo activity, the rapid emergence of resistance, insufficient host range, or the presence of undesirable genetic elements within the phage genome. Targeted genetic editing of wildtype phages (phage engineering) has successfully been employed in the past to mitigate some of these pitfalls, and to increase the therapeutic efficacy of the underlying phage variants. Clearly, there is a large potential for the development of novel, markerless genome editing methodologies to facilitate the engineering of therapeutic phages. Steady advances in synthetic biology have facilitated the *in vitro* assembly of modified phage genomes, which can be activated ("rebooted") upon transformation of a suitable host cell. However, this can prove challenging, especially in difficult-to-transform Gram-positive bacteria. In this chapter, we detail the production of cell wall-deficient L-form bacteria and their application to activate synthetic genomes of phages infecting Gram-positive host species.

Key words: Bacteriophage engineering, L-form bacteria, synthetic biology, phage therapy

1 Introduction

General overuse and prescription of antibiotics in agriculture and healthcare throughout the previous century has led to a steady increase in the amount of circulating and newly arising antimicrobial resistant pathogens ***(1, 2)***. Bacteriophages (phages) are a natural alternative to conventional antibiotics and have been used successfully in many compassionate use cases to treat bacterial infections where the current standard-of-care fails ***(3-5)***. The majority of current therapeutic applications utilize natural phages (i.e., genetically wildtype), which can be associated with several shortcomings such as narrow host range, emergence of bacterial resistance to phage infection, phage-mediated horizontal gene transfer of antibiotic resistance, and prophage integration ***(6-8)***. These limitations are mostly a result of phage-host coevolution, which constitutes a complex interplay between factors, including receptor adaptations, host defense mechanisms, and intricate phage-phage interactions ***(9)***. Bacteriophage engineering has successfully addressed some of these hurdles, e.g. by altering or broadening of host range, by removing genetic determinants of lysogeny or through the delivery of antimicrobial effector genes to enhance therapeutic efficacy. Furthermore, through the engineering of lysis-deficient phage, the release of inflammatory bacterial components (such as endotoxin) can be minimized ***(5, 10-15)***. For an overview of phage engineering applications, we refer the reader to ***(16)***. Several bacteriophage engineering methodologies have been published to date and are based either on homologous recombination (HR) or on the assembly and rebooting of synthetic genomes ***(13, 14, 17)***. The key advantage of using HR-based engineering is the ability to edit large phage genomes, which represent a significant fraction of therapeutic phage candidates. However, these methods are laborious and require functional cloning systems within the host organism. Furthermore, cloning can be limited by gene toxicity. In general, HR produces a mixture of wildtype and engineered phages, necessitating a subsequent selection step to identify and isolate the engineered phage. Many

of these challenges can be overcome by assembling modified phage genomes using *in vitro* methods. Such protocols are fast and simple, enable the simultaneous introduction of multiple modifications in a single step, and do not require laborious screening or cloning of potentially toxic genes. *In vitro*-assembled genomes must be activated to allow the production of virus progeny, which can either be achieved within a suitable host cell upon transformation ***(18-20)*** or using *in vitro* transcription/translation systems (TXTL) ***(21-23)***. Most rebooting methods are based on E. coli, and are therefore well-established for Gram-negative bacteria, particularly for phages that infect *Enterobacteriaceae*. Phages that target Gram-positive bacteria are more challenging to modify using synthetic methods (i) because their thick peptidoglycan layer prevents the efficient transformation of assembled genomes ***(15)*** and (ii) because the current cell-free phage production systems are optimized for phages of Gram-negative bacteria ***(24)***. The transformation barrier can be overcome by using bacteria that grow in the absence of a functional cell wall ***(25, 26)***. These so-called L-forms can take up large DNA molecules and have been shown to efficiently reboot wildtype and synthetic phage DNA in response to simple polyethyleneglycol (PEG) chemical transformation, even across genus boundaries ***(20, 27)***. For example, the *Listeria monocytogenes* (*L. monocytogenes*)-derived L-form strain Rev2L reboots several phages that infect Gram-positive hosts ***(20)***. L-forms have been observed across a wide range of bacterial species and could therefore offer a simple solution for the rebooting of phages infecting the most important Gram-positive AMR pathogens. L-forms can be readily obtained by culturing bacteria in an osmotically stable milieu in the presence of high concentrations of cell-wall active compounds, such as β-lactam antibiotics or bacteriophage endolysins ***(28)***. **Fig. 1** shows characteristics of various L-forms grown on solid and liquid medium , as well as images visualizing key microscopical features. Several caveats are associated with L-form rebooting. Firstly, not all L-forms reboot genomic phage DNA and not all phage genomes can be rebooted in a given L-form. In **Fig. 1**, we demonstrate this limitation using three L-form strains derived from

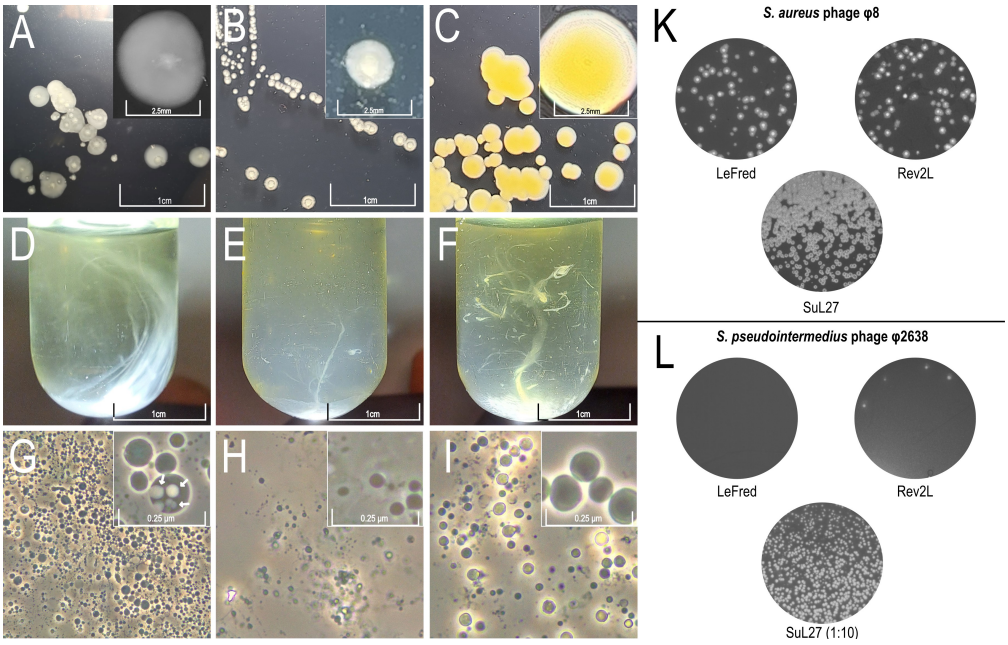

Figure 1: Cell-wall deficient L-form bacteria.

Panels (**A-C**) show characteristic "fried-egg" morphology of L-form colony growth on antibiotic-supplemented DM3 agar plates for *S. aureus* (**A**), *L. monocytogenes* (**B**) and *S. xylosus* (**C**)-derived L-form variants. (**D-F**) show varying, filament-like growth characteristics of *S. aureus* (**D**), *L. monocytogenes* (**E**) and *S. xylosus*-derived (**F**) L-forms in antibiotic-supplemented DM3 liquid culture. Using microscopic imaging (**G-I**) one can distinguish morphological differences between *S. aureus* (**A**), *L. monocytogenes* (**B**) and *S. xylosus* (**C**)-derived L-form variants. The propagation via intracellular vesicle formation often observed in L-form bacteria (***28***) is visible (**G**, white arrows). (**K**) shows results of the rebooting of *S. aureus* bacteriophage ϕ8 genomic DNA in *S. aureus* (LeFred), *L. monocytogenes* (Rev2L) and *S. xylosus* (SuL27)-derived L-form variants. For each rebooting reaction, 500 μl supernatant were plated on the corresponding phage propagation host. Each visible plaque in the bacterial lawn is indication of a successfully rebooted, viable phage particle. (**L**) shows corresponding results for *S. pseudointermedius* phage ϕ2638 (50 μl supernatant were used for LeFred and Rev2L, 5 μl for SuL27).

L. monocytogenes, *Staphylococcus xylosus*, and *Staphylococcus aureus*. The ability of an L-form to efficiently reboot a given phage genome is likely linked to phage-host compatibility factors, such as codon-usage, promoter compatibility, and host-specific DNA modifications. Although cross-genus rebooting of phage DNA has been observed, in our experience, the efficiency of genome activation diminishes with phylogenetic distance. It is, therefore, advisable to isolate new L-form strains for the target species of interest. Here, we present the procedural workflow used for the development of stable, culturable L-form bacteria capable of rebooting synthetic phage DNA to form viable, infectious phage progeny.

2 Materials

The protocol is presented with *S. aureus* strain SH1000ΔmutS as an example strain. Species-specific media, cultivation conditions, or other variations are indicated where applicable. Solutions and reagents should be handled and stored at room temperature unless indicated otherwise. Analytical grade reagents and ultrapure water (Milli-Q or any other water meeting purity standards of >= 18.2 MΩ cm^{-1} at 25°C) should be used unless stated otherwise. All buffers and media should be sterilized by autoclave at 121°C for 15 min unless stated otherwise.

2.1 Antibiotics-induced L-form conversion

1. *S. aureus* strain SH1000ΔmutS (*see* **Note 1**) as -80°C frozen stock (25% glycerol (v/v)).

2. Brain-heart infusion (BHI) nutrient broth: 37 g/L BHI in water, pH 7.4. Weigh 37 g of BHI (powder form) into a graduated cylinder and fill up to a volume of 1 L (*see* **Note 2**). Mix using a magnetic stir bar and adjust the pH to 7.4 (*see* **Note 3**).

3. BHI agar plates: Prepare BHI nutrient broth as described above and add 14 g/L agar to the solution prior to autoclaving (*see* **Note 4**). Pour 25 mL of autoclaved BHI agar per standard 10 cm petri dish.

4. Appropriate bacterial culture container (*see* **Note 5**).

5. DM3 supplement solution: 91.6 g/L succinic acid, 7.7 g/L glucose, 5.4 g/L K2HPO4, 2.3 g/L KH2PO4, 6.3 g/L MgCl2.6H2O in water (pH 7.3). For 1 L of medium, begin by adding ∼400 mL water to a >1 L graduated cylinder. Add 91.6 g succinic acid and adjust the pH to 7.3 using NaOH (10M) (*see* **Note 6**). Add the remaining ingredients, fill up to 1 L with water and stir until all components have fully dissolved. Sterilize by sterile filtration (0.22 μm micropore filter) (*see* **Note 7**).

6. DM3 nutrient broth: 14.29 g/L casein peptone (tryptone), 14.29 g/L yeast extract in

water. Dissolve 5 g casein peptone (tryptone) and 5 g yeast extract in 350 mL water (*see* **Note 8**).

7. Osmotically stable DM3 medium: 350 mL DM3 nutrient broth (from step 5), 645 mL DM3 supplement solution (from step 4), 5 mL bovine serum albumin (BSA, 2% (w/v)). Mix 645 mL DM3 supplement solution with 350 mL DM3 nutrient broth. Add 5 mL of sterile filtered BSA (2% (w/v)) (*see* **Note 9**).

8. DM3 agar plates: Prepare DM3 nutrient broth and supplement solution as described above, except that 8 g/L agar are added to the nutrient broth prior to autoclaving (*see* **Note 10**). Furthermore, the supplement solution must be warmed to ∼50-60°C before mixing with the autoclaved DM3 nutrient broth agar to prevent the agar from congealing before plates can be poured. Add antibiotics (if specified) once the agar has cooled to ∼60°C, mix gently by inverting the flask and proceed immediately to pouring 25 mL per standard 10 cm petri dish.

2.2 DNA synthesis and assembly

1. Designed primer pairs (*see* **Section 3.2**)

2. DNA polymerase/PCR-kit compatible with the specific phage/bacterium genome characteristics such as G/C content.

3. Gel electrophoresis setup.

4. PCR purification kit of choice.

5. Gibson isothermal DNA assembly reagent (e.g., GeneArt™ Gibson Assembly HiFi Master Mix)

2.3 L-form rebooting and phage recovery

1. Assembled phage DNA (see Section 3.2).
2. Solubilized Penicillin at appropriate stock concentrations (e.g., 200 mg/mL).
3. Spectrophotometer for OD_{600} turbidity measurement.
4. Falcon™ 50 mL Conical Centrifuge Tubes or equivalent.
5. Polyethylene Glycol (PEG) 20000 (40% (w/v)) in water.
6. Suitable bacterial phage propagation host as a cryostock or plate streak.

3 Methods

All procedures should be carried out at room temperature unless stated otherwise. Cultivation conditions of 37°C and BHI nutrient media pertain to *S. aureus* specifically and should be adapted for other bacterial species accordingly.

3.1 Antibiotics-induced L-form conversion

1. Use an inoculation loop to spread SH1000ΔmutS onto a BHI agar plate. Place the plate at 37°C and let grow overnight (*see* **Note 11**).

2. Fill an appropriate aerated bacterial culture container (*see* **Note 5**) with 5 mL BHI broth and inoculate with a single colony from the incubated agar plate. Let grow overnight at 37°C with constant agitation (180 rpm culture shaker).

3. Dip a fresh inoculation loop into the overnight culture and spread the residual liquid onto a DM3 plate (supplemented with 200 μg/mL penicillin) (*see* **Note 12, Note 13**).

4. Wrap the plate in sealing film to reduce moisture loss and store at 37°C.

5. Inspect the plate every few days until colonies displaying the characteristic "fried-egg" morphology of L-form cultures are visible (**Fig. 1A-C**). The cells should also be inspected under the microscope to verify L-form conversion (Fig. 1G-I).

6. Use an inoculation loop to scrape cell material from an L-form colony and spread it onto a fresh DM3 (+200 μg/mL penicillin) plate.

7. Repeat steps 4-6 two more times (*see* **Note 14**).

8. Beginning with passage #3, in addition to transferring picked L-form colonies onto a new plate, also inoculate a 1mL DM3 (+200 μg/mL penicillin) liquid culture (*see* **Note 15**) with scraped cell material (*see* **Note 16**, Fig. 2).

9. Let the culture grow at 37°C without agitation until L-form growth is visually evident (**Fig. 1D-F**).

10. Homogenize the cell suspension by vortexing and transfer 10 μl into a fresh 1 mL

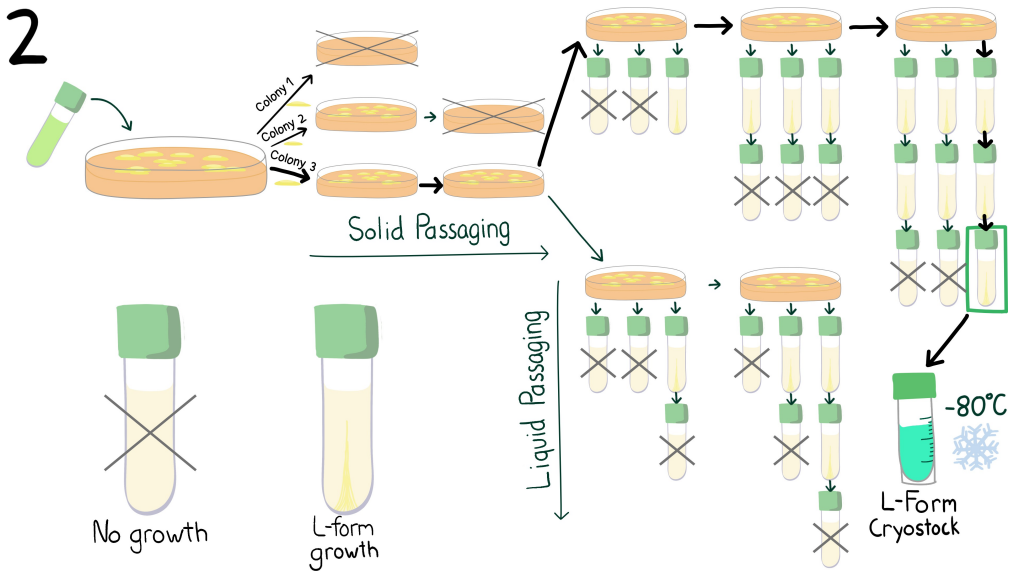

Figure 2: Schematic of one possible variant of the L-form cultivation workflow.

Briefly, DM3 (+Pen200) agar plates are streaked with *S. aureus* wildtype cells then serially passaged, first on solid, then on solid and liquid medium until reliable L-form growth in liquid culture is achieved. Thicker arrows trace the path of a successful series of passages.

DM3 (+200 μg/mL penicillin) liquid culture. Repeat steps 9 and 10 several times (at least thrice or more, **Fig. 2**, *see* **Note 17**).

11. Once reliable liquid growth has been achieved in iterative passages, the homogenized cell suspension should be frozen as a cryostock at -80°C in 25% (v/v) glycerol.

12. Inoculate 5x 1 mL DM3 liquid medium (+200 μg/mL penicillin) from the cryostock (*see* **Note 18**). If L-form growth (OD_{600} >= 0.15) can be achieved reliably from the cryostock, the L-form strain is deemed viable and tested for the ability to reboot phage DNA. This can be done by extracting genomic phage DNA and using it during the rebooting reaction as template DNA, as described in section 3.3. Note that this correlates with but does not guarantee the success of rebooting synthetically assembled DNA of the same sequence.

3.2 DNA synthesis and assembly

1. Design primers to split the phage genome into equally sized fragments with ∼40 bp overlaps at each end (**Fig. 3**, *see* **Note 19 and 20**). Primers should be designed in concordance with the provider's specifications for the polymerase used to amplify the fragments (*see* **Note 21**).

2. Perform polymerase chain reaction to obtain each genome fragment using the previously designed primers and provider-specified PCR conditions. 1 μl of phage lysate can initially be used as template (*see* **Note 22**).

3. Perform gel electrophoresis with each sample following PCR to determine correct, pure amplification of the intended genome fragments (*see* **Note 23**).

4. Perform PCR purification with the obtained PCR products prior to genome assembly (*see* **Note 24**).

5. Assemble the genome fragments using Gibson isothermal DNA assembly reagent according to the provider's specifications (*see* **Note 25**).

3.3 L-form rebooting

1. Pre-warm DM3 liquid medium to 37°C (*see* **Note 26**).

2. Add 200 μg/mL Penicillin to the pre-warmed DM3.

3. Distribute 1 mL of supplemented DM3 (+Pen200) into sterile glass culture vials (*see* **Note 5**, **Note 27**).

4. Inoculate each aliquot with L-form from the cryostock using a sterile inoculation loop (*see* **Note 18**).

5. Mix gently and place at 37°C (without agitation) for 18-32 h (*see* **Note 28**).

6. Disperse the aggregated L-forms by vortexing until the culture appears homogenous (*see* **Note 29**).

7. Determine the OD_{600} of the culture and adjust to OD_{600} = 0.15 using pre-warmed DM3 medium.

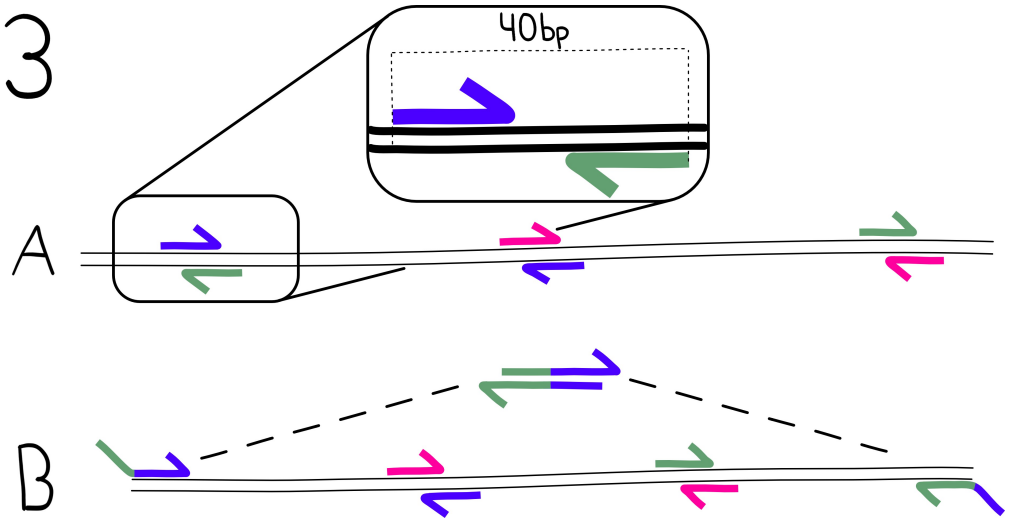

Figure 3: Cell-wall deficient L-form bacteria.

Schematic of primer pair placement (colored arrows) on the bacteriophage genome (parallel black bars). For circularly permuted, terminally redundant phage genomes (**A**), primer pairs can be positioned arbitrarily on the sequence, however we recommend roughly equidistant primer pairs and overlapping placement at fragment junctions of at least 40 bp. Panel (**B**) shows the appropriate placement of terminal primers for fixed-end bacteriophage genomes and their complementary 5' synthetic overhangs (colored appendages of the terminal primers).

8. Pipette 100 μl L-form culture (OD_{600} = 0.15) into a 50 mL canonical centrifuge tube or similiar.

9. Add the assembled DNA (*see* **Note 30**) and mix thoroughly by pipetting.

10. Add 150 μl 40% PEG (w/v) and mix thoroughly by pipetting (*see* **Note 31**).

11. Incubate at RT for 5 min.

12. Add 10 mL 37°C pre-warmed DM3 medium (without antibiotics) and mix gently.

13. Incubate at 37°C for 18-24 h without agitation (*see* **Note 28**).

14. Prepare a culture of a host compatible with the rebooted phage in preparation for phage recovery (*see* **Note 5**, **Note 32**).

3.4 Phage recovery

1. Perform soft-agar overlays of the L-form supernatant using the phage propagation host and incubate plates overnight at 37°C. If rebooting was successful, rebooted phages contained in the supernatant should form plaques on the propagation host (*see* Note 33).

2. Individual plaques formed on the bacterial lawn with high likelihood stem from rebooted phage particles and can be isolated and propagated for downstream application (*see* **Note 34**).

4 Notes

1. We have achieved L-form conversion for a number of different bacterial species, including *S. aureus*, *L. monocytogenes* and *S. xylosus*.

2. ddH20 can be used instead of MilliQ in this step. To avoid the very fine nutrient powder dispersing into the air while filling the graduated cylinder, add a small amount of water (e.g., 100 mL) to the cylinder prior to adding the powder.

3. Concentrated HCL and NaOH (10 M, lower concentrations can be used for fine adjustments) are used to adjust the pH.

4. It is advisable to add the agar directly to the flask to be autoclaved to avoid agar clumps adhering to the graduated cylinder when transferring the solution into the flask. Also, adding a magnetic stir bar to the flask prior to sterilization allows for easier dissolution of the agar after autoclaving and prior to pouring.

5. E.g., a 20 mL glass vial with a loose aluminum lid for aerobic growth conditions.

6. Succinic acid is a weak acid, and titration with a strong base such as NaOH leads to a very steep sigmoidal curve at pH 7.3. Thus, when nearing pH 7.3 during titration, the more drastic the change in pH is for the same volume of NaOH added. Add smaller volumes or lower concentrated NaOH to not shoot beyond pH 7.3. Alternatively, titration with a weak base is possible, but has not been tested.

7. It is recommended to use a large-volume micropore filter in combination with a vacuum pump for large volumes.

8. Preparing precisely 350 mL DM3 nutrient broth in a 1 L flask will allow us to fill up to 1 L with DM3 supplement solution after autoclaving.

9. The final concentration of BSA in the DM3 medium is 0.01% (w/v).

10. This refers to the agar concentration in the final medium solution (after adding DM3 supplement solution). I.e., the concentration during autoclaving is ∼23 g/L (8 g/350 mL).

11. *S. aureus* can also be grown at room temperature with ∼3-fold reduction in growth speed (can be advantageous for culturing over the weekend).

12. If L-form growth cannot be achieved using a specific combination and concentration of antibiotics, variations should be attempted (e.g., 500 μg/mL fosfomycin or other cell-wall active antibiotics at variable concentrations and combinations). We advise determining the MIC for each host/antibiotic combination.

13. DM3 agar plates are much softer than conventional 14 g/L plates and the surface can be easily penetrated, which is to be avoided. Spread the bacterial cells carefully. Alternatively, several μl of the overnight culture can be pipetted onto the plate and dispersed using a conventional cell spreader.

14. The passage number is from experience and likely arbitrary. The number of passages needed to establish stable L-form growth has not been systematically quantified and may vary significantly between bacterial species.

15. Depending on the aeration of the culture vial and the corresponding amount of medium evaporation, this volume may be scaled up to 5 mL.

16. It is not feasible to conduct the passaging in a systematic manner since the parallel passaging of liquid and solid cultures would lead to an exponential increase in the number of samples. See passaging scheme in **Fig. 2**.

17. The rationale behind this iterative passaging is the accumulation of mutations and/or metabolic changes, which result in a stable, fast-growing L-form phenotype over time.

18. Depending on the density of the culture prior to freezing, increased amounts of cell material may be needed to inoculate the liquid culture to ensure reliable growth.

19. In our experience, more than 10 fragments and/or fragments longer than 12 kB in length have proven difficult to assemble, showing exceedingly low yields of rebooted genomes and limiting our success to phage genomes up to ∼80 kB. These parameters, as well as the optimal length of the fragment overlaps should be designed in concordance with the specifications of the DNA assembly kit used.

20. For circularly permuted phage genomes, the overall placement of primer pairs on the genome is arbitrary. For phages with a constant genome structure (cohesive ends or non-permuted terminal redundancy), the precise physical structure must be known to design primers that allow for artificial circularization of the genome prior to transformation (*see* **Fig. 3B**).

21. The polymerase of choice depends on the bacterial species in question and usually depends on factors such as G/C content, potential for secondary structure formation, DNA modifications, etc.

22. The optimal template amount depends on the phage in question and can initially be adjusted simply by diluting the lysate. We recommend purifying genomic phage DNA to quantify the amount of PCR template used.

23. If PCR impurities (i.e., unspecific bands during gel-electrophoresis) cannot be resolved by troubleshooting the PCR reaction, preparative gel extraction can be used to purify fragments. In our experience, visualizing DNA bands with a UV transilluminator can severely damage the DNA. We strongly recommend using a blue-light transilluminator and a suitable dye such as GelGreen.

24. This can be done with a conventional PCR-purification kit. This step is not required by the GeneArt™ Gibson Assembly HiFi Master Mix specifications but is recommended to increase yield.

25. Genome assembly may also be achieved by restriction-ligation but is more laborious and has not been tested.

26. This temperature is also *S. aureus* specific. The temperature should be adapted to the bacterial species in question.

27. Multiple cultures can be set up for redundancy in case of failure of growth but is not stringently necessary if L-form inoculation from the cryostock proves consistently reliable.

28. Times can vary greatly between different L-form and phage variants and should be

adapted accordingly. In case of exceedingly low rebooting efficiency, one can attempt to optimize by varying incubation times prior to (i.e., OD_{600} at inoculation) and/or during rebooting (time post inoculation). Additional variations concerning final PEG concentration, the volume of DM3 addition for PEG dilution, and PEG molecular weight can also be tried. The values used in our protocol have previously been optimized for rebooting of *L. monocytogenes* phages in Rev2L (20). Toxic payloads may also affect rebooting efficiency negatively, particularly if the payload in question is designed to have an intracellular effect.

29. Multiple samples can be pooled prior to vortexing, as long as growth is visible.

30. To maintain osmolarity, this volume should be kept < 20 μl. Larger volumes have not been tested.

31. Wide bore pipette tips make it easier to mix the highly viscous PEG. You can make your own wide bore tips by cutting 1-2 mm off a regular tip using flamed scissors/scalpel.

32. The culture medium should be adapted accordingly if a non-*S. aureus* propagation host is used.

33. Since rebooting efficiency can vary widely between different L-form/phage combinations, in our experience, it has been advantageous to perform the soft-agar overlay with different amounts of L-form solution (for example: 500 μl, 50 μl, 5 μl) to guarantee distinct plaque formation. In some cases, using a large volume of L-form solution can negatively influence the growth of the propagation host during soft-agar overlays.

34. A suitable control consists of rebooting an incomplete assembly (i.e., leaving out one or more fragments for the synthetic phage assembly). Should plaques form from the control reaction, this is an indication of contamination. This could be in the form of foreign bacteriophage contamination or the rebooting of residual genomic phage DNA used as PCR template. The latter can be avoided by including a DpnI digestion of either the PCR or assembly product to remove methylated DNA from the reaction mix.

Conflict of interest statement

S.K. is a part-time employee of Micreos Pharmaceuticals, and M.J.L. is a scientific advisor to Micreos Pharmaceuticals. J.F. and S.M. declare no conflict of interest.

Funding

S.K. and S.M. have been funded through an Ambizione grant (PZ00P3_174108) from the Swiss National Science Foundation (SNSF).

Acknowledgements

We kindly thank Prof. Alex O'Neill (University of Leeds) for providing the SH1000ΔmutS strain used in this study.

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2.4 Manuscript IV: Computational pipeline for targeted integration and variable payload expression for bacteriophage engineering.

Specific contribution by the doctoral candidate

All laboratory experimentation was conducted by the doctoral candidate or by undergraduate students under the direct supervision of the doctoral candidate. Bioinformatic analyses (data aquisition, screening of algorithms and programming) was conducted by the doctoral candidate. The manuscript was written in full- and first-authored by the doctoral candidate. Figures were designed and created by the doctoral candidate. The manuscript was revised by SK.

Computational pipeline for targeted integration and variable payload expression for bacteriophage engineering.

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Keywords: Bacteriophage, genetic engineering, machine learning, expression prediction

Abstract

Bacteriophages are ideal alternatives for bacterial pathogens where conventional antimicrobial treatment has failed. While naturally occurring phages are a viable option for phage therapy, steady advances in synthetic biology and genome engineering allow for targeted alterations to a phage with the potential to improve its function as a phage therapeutic. One such approach is the introduction of antimicrobial genetic payloads. Conventional practice is to integrate such payloads behind genes expressed at very high levels late in the infection cycle, such as the major capsid protein (*cps*). Nevertheless, phages engineered to contain antimicrobial payloads are often difficult to obtain. For example, high expression levels, particularly of intracellularly acting toxic payload proteins, may result in premature arrest of the host metabolism and therefore failure to assemble viable phage progeny. To potentially expand the range of genes viable as genetic payloads, we explored a method to predict expression levels at intergenic loci across the phage genome. Using an available machine learning-based promoter prediction algorithm, *PhagePromoter*, we developed a pipeline to predict loci suitable for the integration of genomic payloads based on estimated promoter locations and strengths across the phage genome. We experimentally validated this approach by engineering phages containing bioluminescent reporter payloads integrated at various predicted loci. We used the well characterized, lytic, *Staphylococcus aureus* infecting bacteriophage, K, as an engineering scaffold and employed homologous recombination to engineer three recombinant phages containing the reporter payload at different predicted loci throughout the genome. The three recombinant phages showed levels of expression in-line with our computational predictions, as well as temporal expression corresponding to the location in early, middle or late gene clusters. In conclusion, although it appears that some integration sites in *Staphylococcus* phages are unviable, our pipeline nevertheless allows for some level of tunable expression of integrated payloads, both via computational pre-

diction of promoter strengths as well as rationale selection of insertion sites based on additional information such as temporal gene expression data.

Introduction

Bacteriophages are ubiquitous viruses which, due to their natural ability to infect and lyse bacteria, have shown potential as alternatives to antibiotics for many decades. Although they were widely superseded by antibiotics, especially in western medicine [1, 2], the steady rise in cases of multi-drug resistant bacterial infections globally has led to the urgent need for alternative treatment options such as phage therapy [3–5]. Despite many case studies showing the successful application of phages to clear antibiotic resistant infections, the use of naturally occurring phages harbors several caveats, including the rapid selection of phage resistant bacteria [2, 6], immunogenicity from prolonged treatment [7] as well as limited host range and the corresponding narrow range of application. To overcome these limitations, as well as enhance the clinical potential of phage applications, numerous methods have been developed to genetically engineer phages with enhanced clinical potential (*see* **Manuscript I** and [1, 8]). Particularly the genetic "arming" of bacteriophages with antimicrobial effector payloads has great potential for the future of bacteriophage engineering in the context of therapeutic applications (*see* **Manuscript V** and [9, 10]). One commonly used approach for the precise, markerless insertion of genetic payloads is homologous recombination either followed by CRISPR-Cas9-assisted counterselection (*see* **Manuscript I** and **Manuscript II**) or screening of plaques for recombinant phages. Conventional practice is to insert payloads behind structural genes expressed late in the infection cycle, which typically show exceedingly high expression levels. Two such genes are the major capsid protein (CPS) and holin/endolysin (Ply) cassette [11–13]. Although this method has proven useful in the past, several caveats exist and could potentially be mitigated through a more diverse selection of insertion loci. For one, post *cps*/*ply* insertion may have unintended effects on transcription and may not always lead to viable phage progeny, something which holds true for all insertion sites, especially for uncharacterized or falsely annotated phage genes. Furthermore, these

locations restrict the temporal expression of the payload to the end of the infection cycle [14, 15]. For specific applications, rapid, early payload expression may be optimal. Reporter phages, for example, express signal payloads which can be used for the detection of pathogens for instance in diagnostic applications, and rapid expression of the payload could allow for faster detection of a signal. Another imaginable approach is to increase payload effect through the rapid and high expression of intracellularly acting compounds early in the infection cycle. Low or intermediate expression levels may also be better suited for payloads where phage amplification at the infection site is beneficial, but high toxicity of the expressed payload may lead to premature cell death and abortion of the infection cycle. To this end, the exploration of insertion sites with variable expression levels at different times in the infection cycle is warranted and the development of approaches to reliably predict potential insertion sites and corresponding expression levels may harbor great potential for future advances in genome engineering and phage therapy.

Information on transcription factor and polymerase binding sites, i.e. promoters, pose a first instance of gene expression regulation and can be inferred from the genetic sequence. Conversely, features such as post-transcriptional/translational modifications can have a significant influence on protein expression, and accounting for these factors for a given locus would necessitate a thorough characterization of the phage prior to engineering. The increasing sophistication of machine learning (ML) algorithms, including those inferring e.g. transcription-factor/polymerase binding sites, as well as the sheer volume and influx of novel bacteriophage sequence data, leads us to believe that it is appropriate to integrate this knowledge into the phage engineering methodology and thereby potentially increase the precision and flexibility of conventional phage-engineering approaches. To this end, we developed and evaluated a tool that predicts payload expression at suitable insertion sites using an ML-based approach for promoter inference and integrated this approach into our phage-engineering pipeline. Homologous recombination (HR) followed by screening for plaques exhibiting bioluminescence was used to engineer three variants

of *S. aureus*-infecting *Kayvirus*, K, each armed with a bioluminescent nanoluciferase (*nluc*) reporter payload inserted at various loci we predicted using our pipeline. K is well characterized in literature, which allowed us to compare our computational predictions and experimental measurements to previously determined gene expression data and experimentally validated promoters. The three engineered phages phages showed predicted expression levels, both in absolute quantity of the expressed payload as well as time to expression based on previously characterized K temporal gene expression data. Overall, the tool developed in this study enables rapid prediction of gene insertion sites with variable promoter strength to tune payload expression levels in therapeutic phage scaffolds.

Results

Comparison of various promoter prediction algorithms

A variety of algorithms exist to date which are designed to predict promoters from genomic sequence data. These range from simple, consensus motif-based approaches [16] to more complex, state-of-the art machine learning algorithms such as support vector machines (SVM), random forests (RF), and convolution neural networks (CNN). We designed our approach to be applicable to whole genome sequences with no limitation on phage/host species, which necessitated that our selected method for promoter prediction fulfilled several pre-defined parameters. To allow for high-throughput processing of multiple genomes simultaneously, the method of choice had to be applicable to complete genome sequences and compatible with, e.g., a multi-fasta input format. Our approach is driven by the hypothesis that machine learning-based probability scores are inherently linked to promoter strengths. We therefore required machine-learning implementations which quantify the probability that a predicted promoter was correctly determined as such. An overview of the various algorithms we tested for seamless integration into our pipeline is given in **Table 1**.

Table 1: **Promoter prediction software and various parameters relevant for seamless integration into our pipeline.**

Tool	Multi-fasta	Big files	Shows promoter core	Score or probability	Output format	Additional notes
Bprom	No	Yes	Yes	Yes	Text on screen	
bTSS-finder	Yes	Yes	Yes	Yes	Text file	Sequence length limited to 500bp. Not executable on MacOS.
BacPP	No	No	No	Yes	Text on screen	

Continued on next page

Table 1: **Promoter prediction software and various parameters relevant for seamless integration into our pipeline.** (Continued)

Tool	Multi-fasta	Big files	Shows promoter core	Score or probability	Output format	Additional notes
Virtual Footprint	No	Yes	Yes	Yes	Text on screen	Requires .gb extension. Sequence length limited to 1000bp.
IBBP	No	Yes	No	Yes	Text file	Windows only
iPro70-FMWin	Yes	Yes	No	Yes	Text on screen	
iPro70-PseZNC	Yes	Yes	No	Yes	Text on screen	Fasta input file requires .txt extension.
iPro54-PseKNC	Yes	Yes	No	Yes	Text on screen	Fasta input file requires .txt extension.
70ProPred	Yes	Yes	No	No	Text on screen	No input file (has to be entered manually on webpage), Webserver persistently unreachable
CNNProm	No	No	No	Yes	Text on screen	
MULTiPly	Yes	Yes	No	No	Text on screen	Slow for large datasets
iPromoter-2L	No	No	No	No	Text on screen	
PromoterHunter	No	No	Yes	Yes	Text on screen	Requires predefined motif matrix
SAPPHIRE	Yes	Yes	Yes	Yes	Text file	
XSTREME	Yes	Yes	Yes	Yes	Text file	
PhagePromoter	Yes	Yes	Yes	Yes	Text file	Trained on validated phage-encoded promoters, *S. aureus* specific

We detail each method's specifications concerning our pre-determined parameters, as well as additional factors which ruled out different methods. Finally, we selected the recently developed, support vector machine (SVM)/artificial neural network (ANN)-based algorithm *PhagePromoter* [17], which was also the sole candidate fulfilling all our requirements. *PhagePromoter* has the added benefit of being adapted towards the prediction of

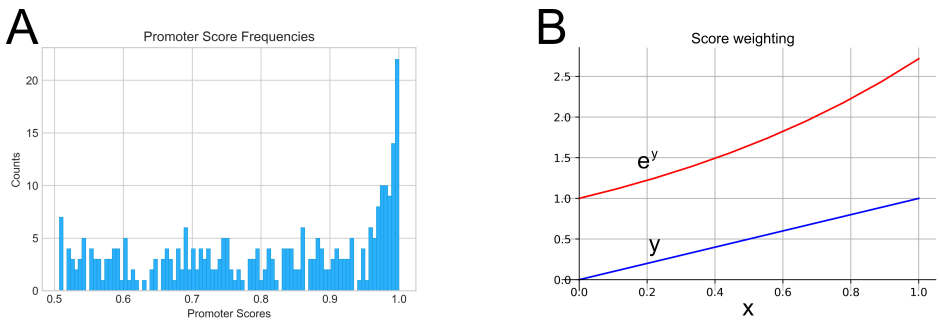

Figure 1. Promoter score distribution and weighting scheme.

(A) Histogram of all predicted promoter scores across the phage K genome. **(B)** Original predicted promoter scores (blue line) were exponentially weighted to favor high scoring promoters (red line).

phage-specific promoters based on experimentally verified, phage-specific input training data.

Promoter prediction and determination of suitable genetic loci for *S. aureus* phage K.

We selected the well characterized, lytic, *S. aureus*-infecting *kayvirus*, K [18], as an engineering scaffold. The annotated, complete sequence record was used (Genbank: KF766114.1). Promoters can overlap with or be encoded within genes, however, we decided to focus mainly on intergenic promoters and excluded those lying within genes for simplicity, albeit allowing for promoters in the first and last 50 bp of each gene. Gene product gp141 of K, which is annotated as two exons with two interspaced genes (gp142 and gp143), was treated as one contiguous stretch to exclude insertion sites between the two exons. Promoter predictions were calculated using the *PhagePromoter* Galaxy Docker build [19]. This resulted in a large number of promoters with a maximum predictive score of 1 **(Figure 1A)**. We therefore selected to classify intergenic regions with a high density of promoter elements (intergenic promoter regions, IPR) and devise a scoring scheme to predict downstream expression levels. IPRs were set as intergenic regions

between annotated genes, but were each allowed to extend 50 bp into the proximal up- and downstream genes. Individual *PhagePromoter* scores were exponentially weighted to place more emphasis on high-scoring predictions **(Figure 1B)**. The IPR score was calculated as the cumulative, weighted scores of all predicted promoters within that IPR. Previous studies have characterized the transcription landscape for K using quantitative mRNA sequencing data [12]. This allowed us to compare our computational predictions to experimental promoter data and gain insight into the validity of our approach. Significant overlap between the experimental data and computational promoter predictions was evident **(Figure 2A)**. As described previously, we hypothesize that variations in the time of expression during an infection cycle may have an effect on the efficacy of certain payloads. The availability of temporal expression data for K [12] **(Figure 2A and B)** motivated us to select high-scoring IPRs for genomic regions expressed at different timepoints during infection. Genes expressed early, late or at an intermediate time during the infection cycle were determined using available data. The highest scoring IPRs, one each for early, late gene and middle gene clusters, were determined. Only IPRs with a proximal downstream gene in the same orientation as the IPR were considered. Insertion sites were selected immediately downstream of the first gene following an IPR. Insertion sites where the intergenic distance was <20 bp were artificially extended to avoid potential disruption the ribosomal binding site (RBS) of the downstream gene. This resulted in 3 IPRs at nucleotide positions 229-518 (early gene), 37505-38806 (late gene) and 119132-119495 (middle gene) **(Figure 2B)**.

Engineered K::*nluc* variants show bioluminescence expression corresponding to predicted promoter strengths and temporal gene expression data.

We performed HR by infecting a K-susceptible host, RN4220 (DSM 26309), into which we had previously introduced the corresponding homology donor plasmid (pEDIT). This

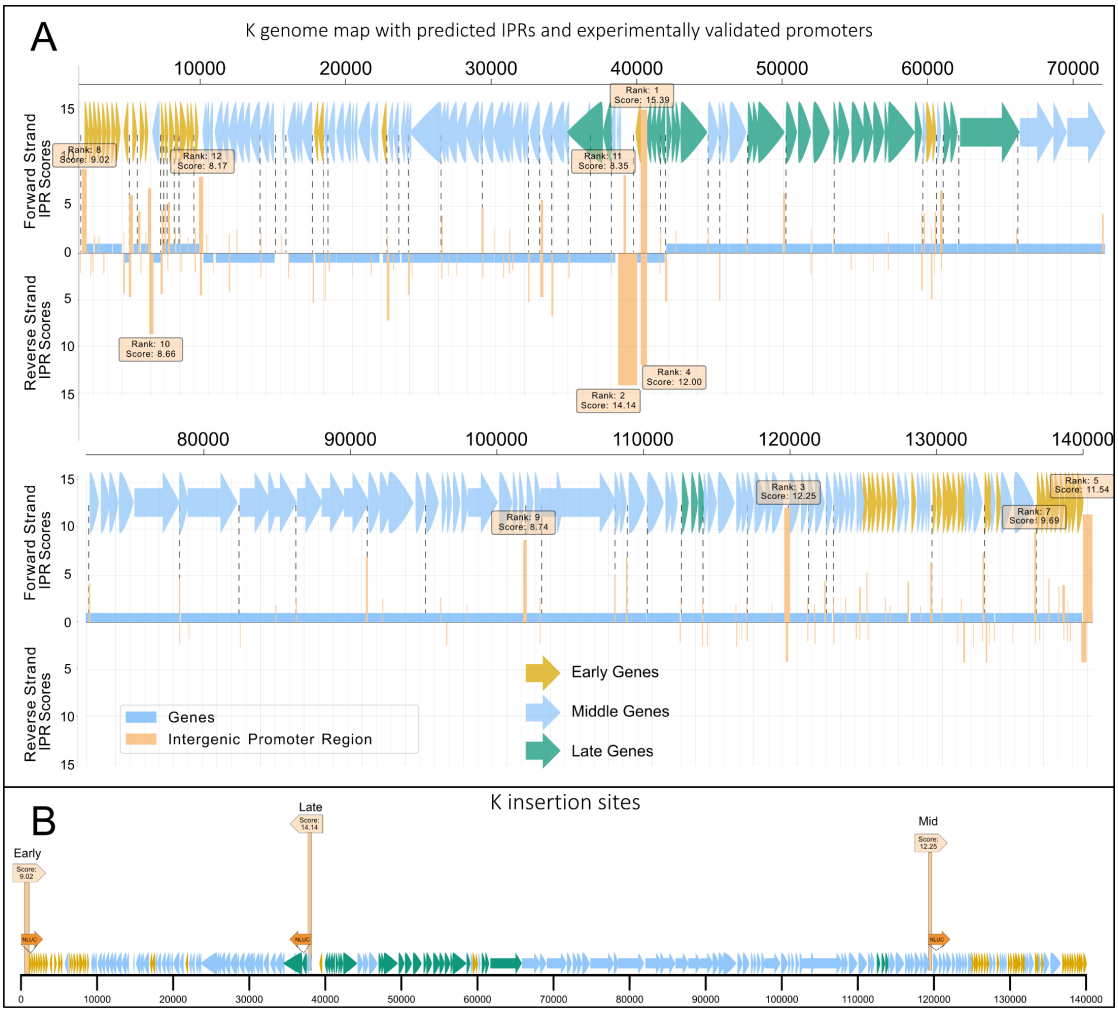

Figure 2. Genome map of phage K, including promoter predictions and selected insertion sites.

(A) K genome map with predicted intergenic promoter regions (IPRs) and experimentally validated promoter scores. Genes are colored based on previously determined temporal mRNA expression and experimentally validated promoters are indicated with vertical dotted lines (adapted from [12]). Depicted below are forward (upper blue bar) and reverse (lower blue bar) oriented genes. All calculated IPRs are shown as vertical orange bars (weighted scores for the 12 highest scoring IPRs are depicted above/below). **(B)** Insertion sites were determined based on the highest scoring IPRs which had a downstream gene behind which insertion of the payload was possible. IPRs with no immediate downstream gene were not considered. One insertion site each was selected for early, middle and late expressed genes.

was done individually for each of the three engineered phages. Bioluminescence measurements were conducted on liquid infection assays with the the K propagation host, *S. aureus* PSK, to determine the presence of recombinant phage, indicated by a significant rise in bioluminescence over time. The assay was repeated with 1:1000 dilutes of positive samples until single, bioluminescent plaques could be detected on full-plate overlays (*see* **Materials and Methods** and **Figure 3A and B**). Plaques were then isolated, purified, validated by PCR and Sanger sequenced. Bioluminescence measurements were performed over a timespan of 140 min at 37°C **(Figure 4A)**. Overall expression levels corresponded with our promoter predictions, with K::$nluc_{\mathrm{Late}}$ (IPR score: 14.14) showing the highest bioluminescence of the three engineered phages, followed by K::$nluc_{\mathrm{Mid}}$ (IPR score: 12.25) and K::$nluc_{\mathrm{Early}}$ (IPR score: 9.02). K::$nluc_{\mathrm{CPS}}$ showed the overall highest bioluminescence, ∼30 times higher than K::$nluc_{\mathrm{Early}}$, although we only predicted one, short promoter element (ATAAAT, PhagePromoter score: 0.921, IPR score: 2.51) upstream of *cps*. The time until onset of expression was exceedingly rapid for all phages, with only K::$nluc_{\mathrm{CPS}}$ showing a significantly slower rise in bioluminescence. To better visualize kinetic differences, we performed the same assay at 25°C for one hour (**Figure 4B**). K::$nluc_{\mathrm{Early}}$ showed the fastest increase in bioluminescence, followed by K::$nluc_{\mathrm{Mid}}$ and K::$nluc_{\mathrm{Late}}$ with similar profiles. K::$nluc_{\mathrm{CPS}}$ showed the longest time until onset of expression.

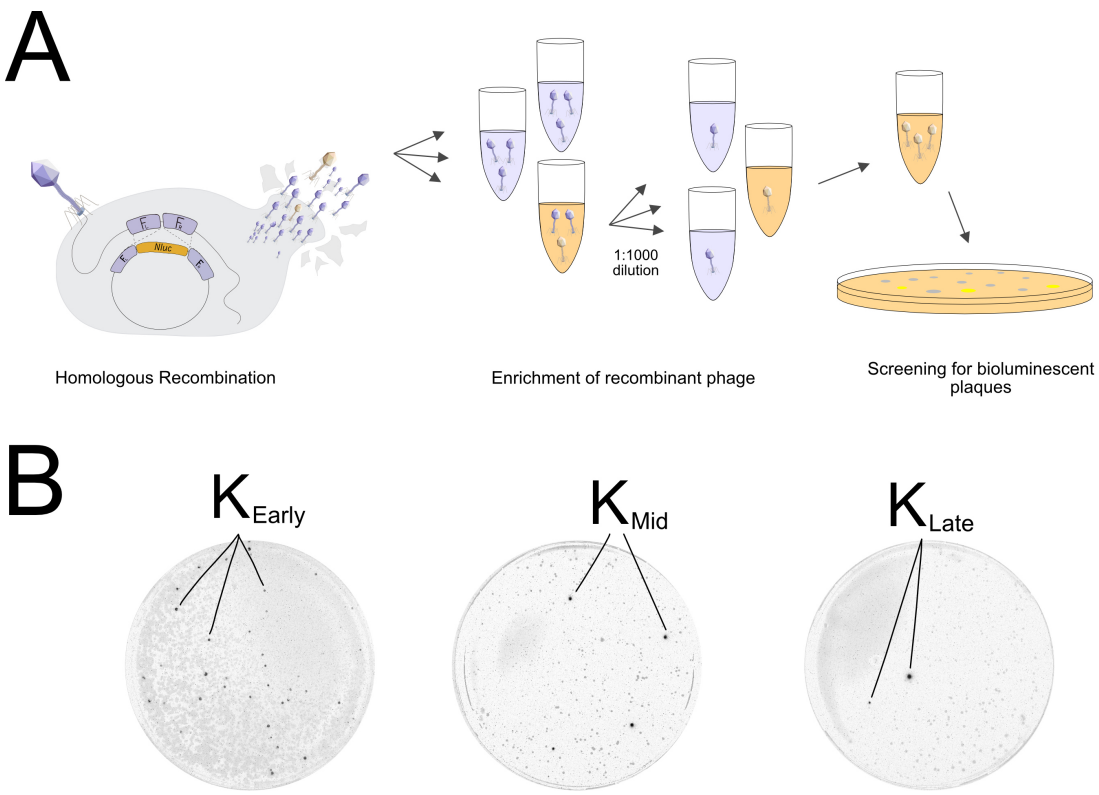

Figure 3. CRISPR counterselection and screening for recombinant K::*nluc* phages.

(A) Lysates obtained from HR were used to obtain escape mutants potentially containing the intended insertion. Lysates were diluted 1000-fold and evaluated for bioluminescence emission in liquid infection assays ($1 * 10^4$ PFU/ml phages (initially), $1 * 10^6$ CFU/ml bacteria). For positive samples, this process was repeated two more times. Finally, plaque assays were performed and individual plaques showing bioluminescence isolated, purified and sanger sequenced to validate correct insertion of the nanoluciferase gene. **(B)** Detection of bioluminescent plaques for phages K::$nluc_{\mathrm{Early}}$, K::$nluc_{\mathrm{Mid}}$ and K::$nluc_{\mathrm{Late}}$.

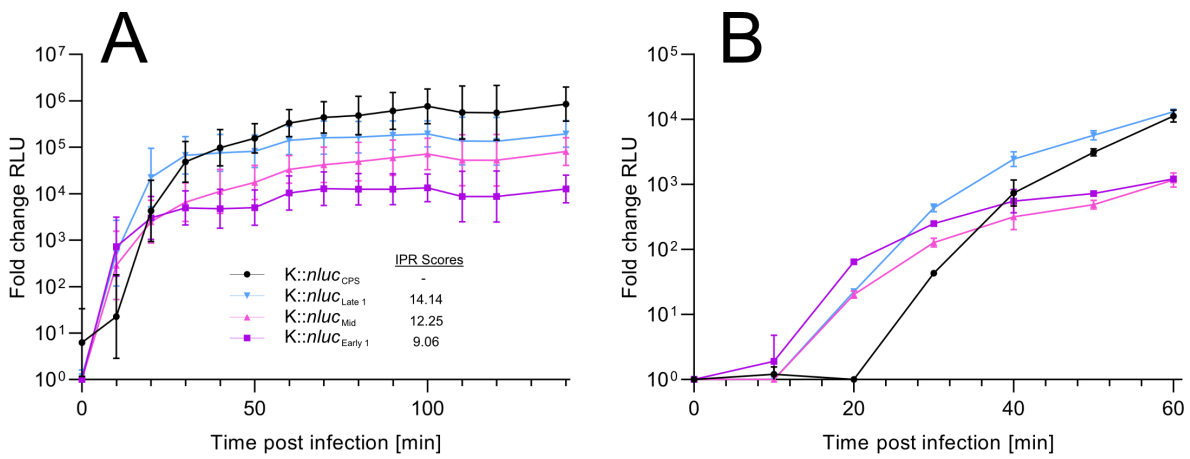

Figure 4. Bioluminescence time course measurements.

(A) Bioluminescence time course measurements for K::*nluc*$_{\text{Early}}$, K::*nluc*$_{\text{Late}}$ and K::*nluc*$_{\text{Mid}}$, as well as a previously characterized K::*nluc*$_{\text{CPS}}$ (*see* **Manuscript II**) were obtained by calculating the fold change in relative light units (RLU) compared to infection with K wildtype at a bacterial density of OD_{600}=0.01 and phage titer of $1 * 10^8$ PFU/ml. K propagation host *S. aureus* PSK was used as a bacterial host and infection occurred at 37°C for 140 min. Values are corrected for background luminescence. **(B)** Bioluminescence time course measurements for phages, bacteria and concentrations described in (**A**) were performed at 25°C for 1h to elucidate differences in temporal expression. (**A**) and (**B**) associated error bars represent mean +/- standard deviation (n=3).

Discussion

Phage engineering has great potential for the targeted genomic alteration of naturally occuring phages to enhance their therapeutic efficacy. The toolkit for phage engineering is steadily growing and a variety of different methods for genetic engineering of phages have been established in recent years (*see* **Manuscript I**). Using these methods, several successful applications using engineered phages have been demonstrated thus far [10, 20, 21]. One factor which has remained largely unaddressed is the localization of a genetic payload within the phage genome. The determination of suitable insertion sites within the genome has remained widely empirical, with conventional practice being the integration behind structural genes expressed late in the infection cycle, such as the major capsid protein [10, 20, 21]. Advances in computational sequence analyses, specifically the implementation of machine learning algorithms to elucidate promoters and transcription factor binding sites from nucleotide sequence data, motivated us to explore methods for determining and evaluating alternative integration sites. To this end, we evaluated a panel of previously published promoter prediction methods and applied the method most suitable to our application. Promoters are short sequence motifs on the genome, typically upstream of the gene whose expression is regulated, and facilitate RNA polymerase (RPo) association and transcription initiation at the transcription start site. Initiation rates and RPo kinetics have been shown to have high sequence dependency with gene expression varying up to 10 000-fold depending on the specific promoter sequence [22–25]. Using a novel phage genome annotation tool, *PhagePromoter* [17], we first predicted promoters and associated scores, clustered adjacent promoters into intergenic promoter regions (IPRs) and attempted to predict sites which exhibit high levels of expression, thus defining sites potentially suitable for payload integration.

The correlation between numerous metrics based on promoter sequence motifs and corresponding gene expression levels has undergone extensive research [26–29], which

motivated us to attempt to use promoter sequences to predict suitable insertion sites and resulting payload expression levels. It has been shown that there is a strong correlation between sequence conservation and promoter strength, implying that promoter sequences close to consensus typically result in stronger RNA-polymerase (Rpo) affinity and therefore transcription levels [29, 30].

Although promoters close to consensus are generally attributed higher expression profiles, recent studies have shown a converse effect of strong RPo binding, with overstabilization of the initiation complex at the transcription start site leading to lower levels of gene expression [31]. This has so far only been shown for small, single nucleotide sequence variations, and it is possible that these effects are limited to genes which already have high baseline expression levels [31]. The general distance to consensus of a promoter is therefore likely still a viable metric for our approach. Particularly since we employ a ML-based approach where specific sequence motifs are correlated with experimental expression levels in the training dataset, these effects may not be as pronounced as conventional, non ML-based consensus methods.

One recent study shows an inherent bi-directionality of promoters, putting into question the traditional assumptions of promoter orientation and transcription initiation [32]. Interestingly, we frequently observed the co-occurrence of predicted promoter regions on both strands, which is possibly a result of this promoter-based DNA-sequence symmetry (**Figure 2A**).

Finally, the low G/C content of *Staphylococcus* species presents an inherent biased for A-T rich stretches and may lead to significant overprediction of TATA motifs, which are characteristic of the -10 promoter region. Also, purine-pyrimidine base preferences have a strong effect on the energetic stability of the DNA double helix, which in turn can have effects on transcription independent of promoter sequence motif conservation [33].

Machine learning approaches are based on large amounts of experimentally validated training data, in our case in the form of bacterial and phage encoded promoters with

corresponding gene expression levels. We hypothesized that the correlation between promoter sequence consensus and expression levels allows us to use ML-generated probability scores (i.e., the probability that the algorithm correctly predicted a promoter) as a proxy for promoter strength. Nevertheless, this assumption does not include potential effects of transcription factors, post-transcriptional regulation and more complex effects of promoter architecture, therefore warranting further analyses into these factors in the future.

Our scoring metric consists of weighting individual promoter scores to emphasize those with high probability and the subsequent grouping of adjacent promoters within intergenic regions into intergenic promoter regions (IPRs). Although we argue that this holistic approach reduces the potential of selecting false positives, the approach is nevertheless rudimentary and may exclude potentially viable insertion sites (i.e., false negatives).

PhagePromoter has the advantage that the training data and output metrics contain information on phage and bacteria-specific promoters. Although the use of phage-specific promoters makes this method ideal for phage engineering, the complexity of phage-host evolution makes it inherently difficult to disentangle evolutionary relationships between promoters and their origins. Furthermore, although the phage-specific nature of *PhagePromoter* may be advantageous for our phage-specific application, we were severely limited in our choice of methods due to our need for the ability to generate high-throughput, full genome promoter predictions as well as the adaptability to different phage/host species. Other algorithms may yield more precise promoter predictions and should be evaluated for suitability for such phage engineering pipelines in future studies.

To experimentally validate these predicted sites, we employed homologous recombination-based (HR) engineering for broad-host range, lytic *S. aureus* phage K to insert a bioluminescent nanoluciferase (*nluc*) reporter payload gene at various predicted loci. Previous engineering efforts using K exhibited no toxicity or size-dependent packaging defects of the *nluc* payload (*see* **Manuscript II**), which motivated us to use

this payload as an easily quantifiable proxy for gene expression. We initially attempted do use CRISPR-Cas9-assisted counterselection (CS) following HR to isolate recombinant phage (*see* **Manuscript II** and **Manuscript V**). We initially also included two additional insertion sites for early and late gene clusters, based on the second-highest scoring IPRs for each cluster (nucleotide positions 136365-136470 (Early 2) and 59907-60036 (Late 2). HR-based, CRISPR-Cas9 CS-assisted engineering of K proved difficult compared to previous studies where an insertion site downstream of the major capsid protein was used (*see* **Manuscript II**). We were able to obtain efficient CS for only 2 of the 5 insertion sites. Recombinant phages for these two integration sites showed disruption of the PAM motif, but lacked the *nluc* gene entirely. This strongly suggests that insertion at these sites results in a severe fitness costs for the phage and does not allow for the production of viable phage progeny. *Nluc* expression, based on the rise in bioluminescence in liquid infection assays using the K propagation host PSK, could be detected in the lysates following HR, although not for the two additional insertion sites for early and late genes, where the second highest IPRs were selected (K::$nluc_{\text{Early 2}}$ and K::$nluc_{\text{Late 2}}$). We therefore performed 1:1000 dilutions and subsequent infection of PSK for the lysates containing K::$nluc_{\text{Early}}$, K::$nluc_{\text{Mid}}$ and K::$nluc_{\text{Late}}$ phages. This process was repeated until bioluminescent plaques could be reliably detected using soft-agar overlay and subsequent imaging using a long exposure time (60s).

We conducted bioluminescence time course measurements with the three succesfully engineered phages K::$nluc_{\text{Early}}$, K::$nluc_{\text{Mid}}$ and K::$nluc_{\text{Late}}$. Expression levels of our engineered phages were in line with the predicted IPR scores, suggesting that our method of promoter prediction is indeed a viable tool for tuning expression strengths of payloads by targeted insertion into the phage genome. Our predicted IPR scores also corresponded with the observation that genes expressed towards the end of the infection cycle show high levels of expression when compared to early genes [11–13]. Finally, by adapting the bioluminescence time course measurements to take place at 25°C over one hour, we were

able to detect kinetic differences of payload expression based on the insertion in early, middle or late gene clusters.

Although much research has been conducted on the characteristics and architecture of sequence-based transcription regulators, much remains to be understood. We hypothesize that the presence of additional factors known to be active in bacterial transcription regulation may have compounded our ability to predict expression strengths with higher accuracy, as well as the failure to predict highly expressed genes such as *cps*. Such factors include transcription factor binding sites, post transcriptional/translational regulation or secondary mRNA sequence structures [34–37]. Furthermore, the lack of certain transcription activators from Gram-positive infecting phages suggests the presence of additional regulators or mechanisms involved in gene activation [38].

We demonstrate the potential of incorporating state-of-the-art machine learning software into classical sequence analysis to make rational design decisions in the phage-engineering process. Although we benchmarked our method using *PhagePromoter*, the pipeline is inherently modular and other promoter prediction methods could easily be integrated as an alternative or additional validation. We envision this type of pipeline to be of great value for a future of high-throughput, automated phage engineering and that it can contribute to the field by opening the possibility of sequence-based expression prediction for targeted payload insertions.

Materials and Methods

Bacterial strains and culture conditions

S. aureus laboratory strain RN4220 (DSM 26309) was used as an engineering host to generate all K::*nluc* variants. PSK was used as propagation host for K and all K::*nluc* variants. *E. coli* XL1-blue MRF' (Stratagene) was used for plasmid amplification prior to transformation of RN4220. *E. coli* were grown in Luria-Bertani (LB) liquid medium (3M NaCl, 10g/l tryptone, 5g/l yeast extract) overnight at 37°C (180 rpm). *Staphylococcus* species were grown in Brain Heart Infusion (BHI, Biolife Italiana) broth overnight at 37°C (180 rpm).

DNA amplification

Reaction mixes consisted of 19 μl H2O, 2.5 μl of forward and reverse primer respectively, 1 μl template and 25 μl 2x Phusion High-Fidelity PCR Master mix with HF buffer (ThermoFisher). The template amount was varied in cases where fragment amplification proved difficult. PCR reactions were conducted with the following conditions: 5 min at 95°C, 30 sec at 95°C, 30 sec at annealing temperature, (30 sec per 1000 bp) at 72°C, 5 min or 10 min at 72°C. Steps 2 to 4 were repeated for 30-35 cycles. The annealing temperature was varied in cases where fragment amplification proved difficult. Gel electrophoresis was performed in 1% agarose gel (1% agarose in 1x TAE buffer) at 110 V. Samples were loaded as follows: 9 μl H2O, 2 μl 6x DNA loading dye (Thermo Fisher) and 1 μl DNA (from PCR). As DNA ladder, GeneRuler 1 kb DNA ladder was used. The loading dye was supplemented with 100x GelRed™ Nucleic Acid Gel Stains (Biotium).

Bioluminescence assays

For expression analysis of the purified recombinant phages, stationary phase bacterial cultures were diluted to OD_{600} 0.01, inoculated with $1*10^8$ PFU/ml phage and incubated

at 37°C (180 rpm agitation). Bioluminescence measurements were taken by combining 25 μl of the sample solution with an equal volume of prepared buffer-reconstituted nluc substrate as detailed by the manufacturer (Nano-Glo Luciferase Assay System; Promega). Measurements were taken every 10 min in Nunc™ F96 MicroWell™ 446 plates using a GloMax® navigator luminometer (Promega) with 5s integration time and 2s delay. To determine the background-corrected fold-change in relative light units (RLU) for each phage, measurements were normalized to a control reaction of each phage in BHI medium and the fold-change was calculated as the difference in RLU to an infection of the same strain with wildtype K. All measurements were performed in triplicate. To determine plaques containing recombinant, nanoluciferase expressing phage from full plate overlays, 500 μl of Nano-GloR substrate were spread onto the plate. Plates were photographed using Gel Doc XR+ Gel Documentation System, once with no illumination and 50 s exposure time, and once again using trans-white illumination and exposure of 0.2 s. Images were overlayed to determine the location of plaques showing bioluminescence on the full plate. For those engineered phages where no bioluminescent plaques were detectable, enrichment steps were conducted by liquid infection of PSK at an initial MOI of 0.01 ($1 * 10^4$ PFU/ml phages, $1 * 10^6$ CFU/ml bacteria) until a significant rise in bioluminescence ($> 10^3$ RLU) was detectable. The solution was then diluted 1:1000-fold and enrichment repeated. This was continued until bioluminescent plaques were detectable in the plaque assay.

Plasmid construction

Design of homologous recombination (HR) and CRISPR-counterselection (CS) plasmids were done analogous to **Manuscript II**. The plasmid pLEB579 (kindly gifted by T. Takala, University of Helsinki, Finland) was used as a backbone for both the plasmid used as an editing template (pEDIT) and the CRISPR-Cas9-counterselection (CS) system (pSELECT). We used an *S. pneumoniae*-derived dual-RNA:Cas9-based CRISPR

system [39] reprogrammed to cleave wildtype K at two loci flanking the payload insertion site. For the construction of these pSELECT plasmids, we exchanged the two sequences of short CRISPR RNA (crRNA) spacers to target 30 bp stretches of DNA with proximal protospacer adjacent motifs (PAM), up- and downstream of each intended insertion site, respectively. To allow for Cas9 escape of successful recombinant phages, we identified PAMs where the second or third positions could be altered to a non-G nucleotide, while retaining the corresponding amino acid sequence (i.e., synonymous point mutation). A python script was written to automatically select two CRISPR-Cas9 targeting sites for each previously determined location; one up- and one downstream of the insertion site. The exhaustive sampling of all possible synonymous PAM mutations allowed us to identify PAMs as close to the insertion site as possible, which is likely to elevate the chance of correct integration during HR. The script was designed in a manner to adhere to species specific codon usage when generating synonymous mutations where possible. The pEDIT homology donor templates were constructed by integrating an *nluc* gene (optimized for *S. aureus* codon usage, avoiding of Rho-independent termination and an added upstream ribosome binding site: GAGGAGGTAAATATAT), flanked by homology arms corresponding to the intended K insertion site, into the linearized pLEB579 backbone. The homology arms were designed to be 200-400 bp in length and where possible to exclude full-length genes of unknown function to mitigate production of potentially toxic gene products during cloning. All synthetic sequences were acquired as GeneArt String DNA Fragments (Thermo Fischer), albeit the spacer sequences for pSELECT, which were ordered as GeneArt Gene Synthesis (Thermo Fischer). Strings and plasmid backbones were amplified by PCR followed by purification with the Wizard SV Gel and PCR Clean-up System (Promega). Plasmids were assembled using isothermal GibsonAssembly® reaction (NEBuilder® HiFi DNA Assembly Master Mix).

Transformation of *E. coli* cells

To prepare electrocompetent *E. coli*, LB medium was inoculated with an overnight culture grown until an OD_{600} of 0.4-0.6 was reached. The cells were incubated on ice for 30 min, centrifuged (4000 g, 15 min, 4°C) and pellets resuspended in 10 % glycerol. This was repeated three times and cells were stored at -80°C if not used directly. DNA samples were transferred onto MF-Millipore 25 μm MCE Membrane Filters (SigmaAldrich) in H2O and removed again after 10-20 min. Electrocompetent cells were mixed with the DNA and electroporated at 2.5 kV, 200 Ω, 25 μF. 1 ml SOC medium (2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 20 mM Glucose) was added immediately and the cells in SOC were left at 37°C for 15 min without agitation, then with agitation (300 rpm) for 1h. Cells were grown overnight on selective medium at 37°C and successful transformants isolated.

Transformation of *S. aureus*

To generate electrocompetent cells, BHI medium was inoculated with *S. aureus* and grown until an OD_{600}=1 was reached. Cells were left on ice for 15 min, then washed three times with cold H2O and two times with 10 % glycerol (3000-5000 g, 10 min, 4°C). The cells were stored at -80°C if not used directly. For electroporation, electrocompetent cells were thawed for 5 min on ice and incubated for another 5 min at RT. They were centrifuged (5000 g, 5 min) and the pellet was resuspended in 70 μl electroporation buffer (10 % glycerol, 0.5 M sucrose). DNA samples were transferred onto MF-Millipore 25 μm MCE Membrane Filters (Sigma-Aldrich) in H2O and removed again after 10-20 min. DNA was then added to the cells and they were electroporated at 2.1 kV, 100 Ω, 25 μF. 1 ml B2 medium (10 g/l casein hydrolysate, 5 g/l D-glucose, 1 g/l potassium phosphate dibasic, 25 g/l NaCl, 25 g/l yeast extract) was added immediately and the cells in B2 were left at 37°C for 2 h with agitation (300 rpm). The cells were then plated onto B2 plates supplemented with selective antibiotics and stored overnight at 37°C. Successful

transformants were isolated the following day.

Plasmid sequencing and evaluation

Sanger sequencing of PCR products (purified with Wizard SV Gel and PCR Cleanup System (Promega)) or plasmids (purified with GenELuteTM Plasmid Miniprep Kit (Sigma-Aldrich) was conducted at at Microsynth AG (Switzerland). Results were analyzed with CLC Genomics Workbench.

Phage handling and storage

Phages were stored in S/M buffer (5.8 g/l NaCl, 2 g/l MgSO4, 6 g/l Tris-HCl (pH 7.5)) at 4°C. To pick individual plaques from plates, wide bore pipet tips were used to transfer a small amount of the plaque-containing soft-agar into 100 μl S/M buffer.

Spot-on-lawn and full plate overlay assays

To perform spot-on-lawn assays, 200 μl of stationary phase bacterial culture were added to 5 ml molten (47°C) LC soft agar (10 g/l tryptone, 7.5 g/l NaCl, 1% D-glucose, 2 mM MgSO4, 10 mM CaCl2, 5 g/l yeast extract, 4 g/l agar), vortexed briefly and poured onto a 0.5x BHI plate. The soft agar was left to dry for at least 20 min at RT. 10 μl spots of 10-fold decreasing phage dilutions were placed onto the agar. The spots were left to dry for 1 h and then stored overnight at 37°C. Full plate overlay assays were performed in a similar manner, with the difference that for each phage dilution, 10μl were added to 5 ml molten LC soft agar together with the host bacteria and then poured onto a 0.5x BHI agar plate. The bacteria were added first, the LC soft agar tube was vortexed briefly, then the phages were added and transferred to the plate. The plates were dried for at least 30 min and incubated overnight at 37°C.

Phage propagation and purification.

To obtain a pure phage lysate, full overlays were performed with the phage on its host bacterium to obtain 3-6 plates with semi-confluent lysis. On the following day, 5 ml S/M buffer were added to each plate and placed at 4°C for 2-3 h with gentle agitation. The lysate was then collected and centrifuged (10'000 g, 10 min, 4°C) to pellet cell debris. The supernatant was sterilized by filtration using 0.22 μm filters. Open-Top Thinwall Ultra-Clear Tube (Beckman Coulter) were loaded with the following CsCl density layers: 1.7 g/ml CsCl, 1.5 g/ml CsCl, 1.35 g/ml CsCl. The densities were obtained by dissolving CsCl salt in S/M buffer. As the topmost layer, the phage lysate sample adjusted to a density of 1.15 g/ml was added. Ultracentrifugation was performed at 20'700 rpm for 2 h at 10°C. The tubes were then collected and the visible phage band was recovered. The lysate was dialyzed for a minimum of 2x 2 h using the Slide-A-LyzerTM Mini Dialysis Device (Thermo Scientific).

CRISPR-Cas9-assisted engineering of K::*nluc*

CRISPR-Cas9-assisted engineering of K::*nluc* was done analogous to **Manuscript II**. Briefly, for each engineered phage, the corresponding pEDIT and pSELECT plasmids were transformed into *S. aureus* RN4220 to acquire the engineering strains RN4220 (pEDIT) and RN4220 (pSELECT), respectively. RN4220 (pEDIT) was infected with serial dilutions of K via soft-agar overlay to obtain a high titer lysate by washing semi-confluent plates with S/M buffer as described above. The assay was repeated with the resulting lysate and RN4220 (pSELECT) as host. Individual plaques were picked from the plates showing the lowest number of (non-zero) PFU and resuspended in SM buffer. PCR amplification using primers flanking the insert site was performed and products showing a size indicative of the intended insertion purified and Sanger sequenced (Microsynth AG, Balgach, Switzerland) to validate the correct genomic sequence.

Bioinformatics

Evaluation of the various promoter prediction programs was conducted with the software versions as they were online available in the time between 02.06.2022 and 08.06.2022. The full genome sequence and annotations of K were acquired from genbank file KF766114.1. The PhagePromoter Galaxy Docker Build (Galaxy Version 0.1.0) was used for generating promoter predictions using the following parameters: Search both strands yes, threshold 0.5, phage family *siphoviridae*, host bacteria genus *Staphylococcus* aureus, phage type virulent. Programming, plotting and sequence analyses were done in Jupyter notebook version 6.1.4 running python 3.8.5. CLC Genomics Workbench version 20.0.4 was used for additional sequence analyses such as primer and string design as well as evaluation of sanger sequencing results.

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2.5 Manuscript V: Engineering of large, lytic *S. aureus* phage K for the expression of antimicrobial effector payloads.

Specific contribution by the doctoral candidate

All laboratory experimentation was conducted by the doctoral candidate or by undergraduate students under the direct supervision of the doctoral candidate. The manuscript was written in full- and first-authored by the doctoral candidate. Figures were designed and created by the doctoral candidate. The manuscript was revised by SK.

Engineering of large, lytic *S. aureus* phage K for the expression of antimicrobial effector payloads.

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Keywords: *Staphylococcus aureus*, bacteriophage, genetic engineering, antimicrobials

Abstract

Overuse of antibiotics in medicine and agriculture has led to a surge in the number of circulating antimicrobial resistant pathogens. *Staphylococcus aureus* is of particular concern considering the large number of both community- and hospital acquired infections, as well as the high frequency of newly emerging antibiotic resistant variants. Bacteriophages, or phages, are bacteria infecting viruses and recent advances in the field has allowed for milestone advances in the application of phages as antimicrobial therapeutics. *S. aureus* phages are particularly difficult to engineer, however recent studies show success in engineering a large, lytic *S. aureus* phage, K, which displays a broad host-range against a large panel of *S. aureus* strains. In this study, we screened a variety of payload genes that could enhance target-specific killing via phage-mediated delivery and production of toxic, intracellularly-acting proteins. Although the majority of payload genes proved unviable, we successfully obtained two recombinant phages containing a gene coding for an *S. aureus* protein toxin, *mazF*, and the short leaderless bacteriocin-coding gene *lnqQ*. Both engineered phages showed significantly enhanced inhibition of bacterial growth compared to the wildtype when tested against a variety of *S. aureus* strains, including vancomycin-resistant clinical isolates.

Introduction

Stemming from the vast abundance and over prescription of antibiotics in modern society, there is a large and steady increase in the number of bacterial pathogens exhibiting resistance against conventional antibiotics [1]. The need for novel antimicrobials is greater than ever, and the number of newly discovered and approved antibiotics is rapidly declining [2–4]. *Staphylococcus aureus* is one of the most prevalent bacterial pathogens, with up to 50% of humans estimated to be colonized commensally, although this number can be significantly elevated for specific sub-populations, such as health-care workers or hospitalized patients [5]. *S. aureus* can transition from commensal to pathogenic and cause severe tissue or bloodstream infections which can often be life threating. Furthermore, newly emerging strains exhibiting novel modes of antibiotic resistance even against last-line treatments has led to the classification of *S. aureus* as a pathogen of particular concern by numerous public health agencies [6–8]. Consequently, there is an urgent need for novel treatment options.

Bacteriophages, or phages, are bacterial viruses, which are ubiquitous in the environment and have evolved to be highly efficient in infecting and killing bacteria. Their self-replicating nature, high target specificity and little to no effects on eukaryotic organisms make them an ideal alternative for the treatment of bacterial infections [9]. Furthermore, advances in molecular biology and genomics has allowed for the precise manipulation of bacteriophage genomes to induce a diverse set of alterations, typically designed to enhance the lytic ability against target pathogens (*see* **Manuscript I** and [10]). By introducing genetic payloads into the phage genome, the expression of effector proteins aiding in the killing of specific pathogens can be achieved [10, 11]. The correct selection of a suitable phage scaffold to be used for a specific engineering application is an important factor to consider prior to introduction of a payload, and characteristics such as phage lifecycle, host range and lytic ability have to be taken into close consideration.

Previous characterization of the host range of the large, strictly lytic, *S. aureus* infecting phage K shows successful infection, as characterized by plaque formation, of ∼40 - 70% of tested isolates [12, 13]. Our own experiments using nanoluciferase (*nluc*) as a genetic payload suggest that genome delivery and expression of the payload is achieved in 100% of tested strains (*see* **Manuscript II**), which motivated us to attempt to engineer K armed with antimicrobial effector payloads. To this end, we first selected a variety of effector genes coding for proteins which had previously been shown exhibit strong bacteriostatic or bactericidal activity against *S. aureus* or other Gram-positive and -negative species. Furthermore, we selected payloads with different targets and modes of action, including restriction enzymes, protein toxins from toxin-antitoxin systems (TAS), as well as a short leaderless bacteriocin and a phage-encoded depolymerase. Homologous recombination (HR)-driven, CRISPR-Cas9-assisted counterselection (CS) was used to engineer K variants containing the various payloads, analogous to the introduction of the *nluc* payload in **Manuscript II**. The vast majority of recombinant phages could not be obtained. In most cases, we failed to transform *S. aureus* RN4220 with plasmids needed for HR and CS. Furthermore, in cases where our HR and CS systems were functional, we were frequently unable to obtain viable phage progeny, likely due to strong fitness defects for either the phage or host incurred by high toxicity of the payload. However, we were successful in engineering one short leaderless bacteriocin-coding phage, K::*lnqQ*, and one protein toxin-coding phage, K::*mazF*. We found that both K::*lnqQ* and K::*mazF* significantly enhanced phage-mediated killing, particularly in partially resistant *S. aureus* isolates.

Results

Payload gene selection

A number of antimicrobial payloads were initially selected and tested for viable integration into phage K, as well as potential changes in killing efficacy of the resulting recombinant phage, compared to the wildtype. The payloads were selected from literature based on previously reported or hypothesized antimicrobial activity against *S. aureus*. These included different classes of molecules. Restriction-enzymes induce sequence specific double strand breaks based on short DNA motifs (4 - 11 bp) which results in a high probability of DNA degradation when expressed inside the host cell. The selection of restriction enzymes was based on the number of recognition sites in the genomic sequence of the *S. aureus* strain RN4220 (NZ_CP076105.1) and phage K. We ensured to include restriction enzymes wildly differing in the number of cut sites, ranging from several dozen to more than 5000, as well as no predicted cut sites in the K genome. We also included a number of genetic payloads based on toxin-antitoxin systems (TAS) in our study. The protein toxin from the *S. aureus* TAS *mazEF*, MazF, specifically cleaves single-stranded RNA molecules and overexpression has been shown to lead to growth arrest in *S. aureus* [14]. The YoeB1 and YoeB2 toxins of the *S. aureus* TAS *yoeB1-yefM1* and *yoeB2-yefM2* (previously identified as *axe1-txe1* and *axe2-txe2*) are ribosome dependent RNases that cleave close to the start codon and have also been shown to lead to growth arrest in *S. aureus* by inhibiting translation initiation [15, 16]. Additionally, we selected a *Streptococcus pneumoniae* TA-system, *phd-doc*, where overexpression of the Doc toxin has been shown to suppress cell growth in the natural host through obstruction of ribosomes during translation [17]. We additionally included the short leaderless bacteriocin lacticin Q (LnqQ), which has been shown to exhibit bactericidal activity against a variety of Gram-positive bacteria through disruption of the integrity of the bacterial cell membrane [18–20]. Finally, we also included the depolymerase Dpo7, which is a phage pre-neck appendage and

leads to biofilm dispersion through degradation of the polysaccharide components of *S. aureus* biofilms [21]. The selection of antimicrobial payloads used as well as associated characteristics are given in **Table 1**.

Payload gene engineering

We used an HR-based approach followed by CRISPR-Cas9-assisted CS to insert all payloads individually into K (*see* **Manuscript II** and **Figure 1A**). We made efforts to immunize host cells used during the engineering process for all payloads where immunity genes (such as the antitoxin in a TAS) were known. This was done by cloning said genes behind a constitutive promoter on a high-copy plasmid (referred to as pHELP) which we introduced into the HR and CS strains in parallel with the homology donor (pEDIT) and CRISPR system (pSELECT), respectively. For the HR donor template, we employed a plasmid devoid of expression elements such as promoters or enhancers. For all but two payloads, Lacticin Q [19] and MazF [22], we failed to obtain recombinant phage, although problems with the individual payloads arose at different engineering steps throughout the pipeline and are likely differed in their nature. This includes failure to transform the engineering host RN4220 with the immunity plasmid, failure to transform the same strain with the donor plasmid, as well as cases where HR and CS were successful, but the phage did not retain an intact copy of the payload within the genome. The engineering results and potential reasons for failure are listed in **Table 2**.

We were able to obtain plaques for the two phages K::*lnqQ* and K::*mazF*, generated from the two aforementioned payloads, which did not appear to have issues during the engineering pipeline (**Figure 1 B and C**). The the payload insertion site was sequenced to ensure no mutations (besides the two intended PAM mutations) inside or proximal to the gene had occured.

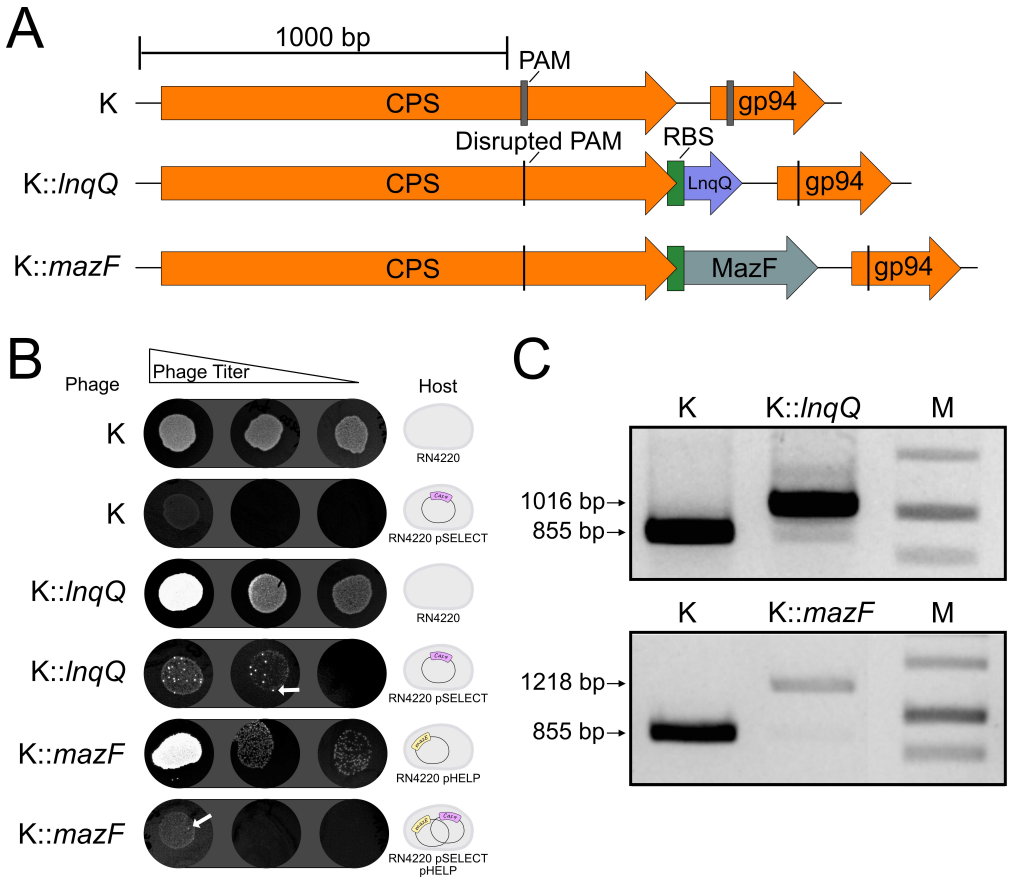


Figure 1: Design and engineering of K::*lnqQ* and K::*mazF*.

(A) Insertion sites of the two successfully engineered phages, compared to wildtype K. Payloads are positioned immediately downstream of the major capsid protein-coding gene (*cps*). A strong ribosomal binding site (GAGGAGGTAAATATAT) was placed before the payload. Two silent point mutations in the homology donor templates disrupt the PAM motif used to select against wildtype phage during counterselection. **(B)** Homologous recombination using the appropriate homology donor strains was performed to obtain K::*lnqQ* and K::*mazF* and plaque formation was subsequently evaluated on the CRISPR counterselection host compared to wildtype K. Phages (K indicates pure wildtype containing lysate, K::*lnqQ* and K::*mazF* indicate mixed lysates following HR) and host cells (RN4220 and the counterselection strain RN4220 pSELECT) used for each plaque assay are indicated to the left and right, respectively. Cells used for HR and CS of K::*mazF* additionaly contained the immunity plasmid pHELP, which expresses the immunity factor MazE. White arrows indicate likely escape mutants which were picked and evaluated via PCR for the correct insertion size. **(C)** Gel electrophoresis of PCR fragments obtained for candidate plaques. The expected PCR fragment sizes for wildtype K and recombinant K are indicated.

Phenotypic analysis of K::*lnqQ* and K::*mazF*

To assess the effect of the payload gene on the infectivity of K::*lnqQ* and K::*mazF* in the K propagation host *S. aureus* PSK (ATCC 19685), we first compared the morphology of plaque formation on a solid PSK bacterial lawn. We could visually not determine any significant difference in plaque morphology between the two engineered phages and the wildtype **(Figure 2A)**. We next determined potential differences in *in vitro* killing efficacy between the three phages. Next to *S. aureus* PSK, we selected three additional hosts who's susceptibility to K we previously quantified using K::*nluc* (*see* **Manuscript II**). This was done in regards to two measures. For one, we determined the efficiency of plaquing (EOP) on a solid bacterial lawn to determine the ability of K to propagate on a variety of *S. aureus* strains. We further measured bioluminescence in liquid infection assays and identified multiple strains where expression of the payload took place based on measured bioluminescence, even though no plaque formation was evident in the EOP assays. This indicates successful genome delivery and payload expression but a failure to complete a full infection cycle and form a visible plaque. This could be due to failure of the phage endolysin to lyse the cell, or premature cell death before assembly of viable phage progeny is completed. To this end, we selected one host which previously showed high EOP and bioluminescence (LI5), as was the case for PSK, and two additional hosts which showed no EOP, but significant levels of bioluminescence (LS5 and LI11) (**Figure 2B**). We performed liquid infection assays with the three phages K, K::*mazF* and K::*lnqQ*, and measured the reduction in turbidity over 24 h **(Figure 2C-F)**. Infection of PSK and LI5, which both previously showed high EOP and bioluminescence, showed a complete control of growth over the period of 24 h for all 3 phages. Conversely, LS5 and LI11, which previously showed no EOP but relatively high bioluminescence, showed significantly increased killing efficiency for the two engineered phage variants. LS5 infection showed no reduction in turbidity for K compared to the growth control, whereas K::*lnqQ* and K::*mazF* showed suppression of bacterial growth for ca. 6 hours

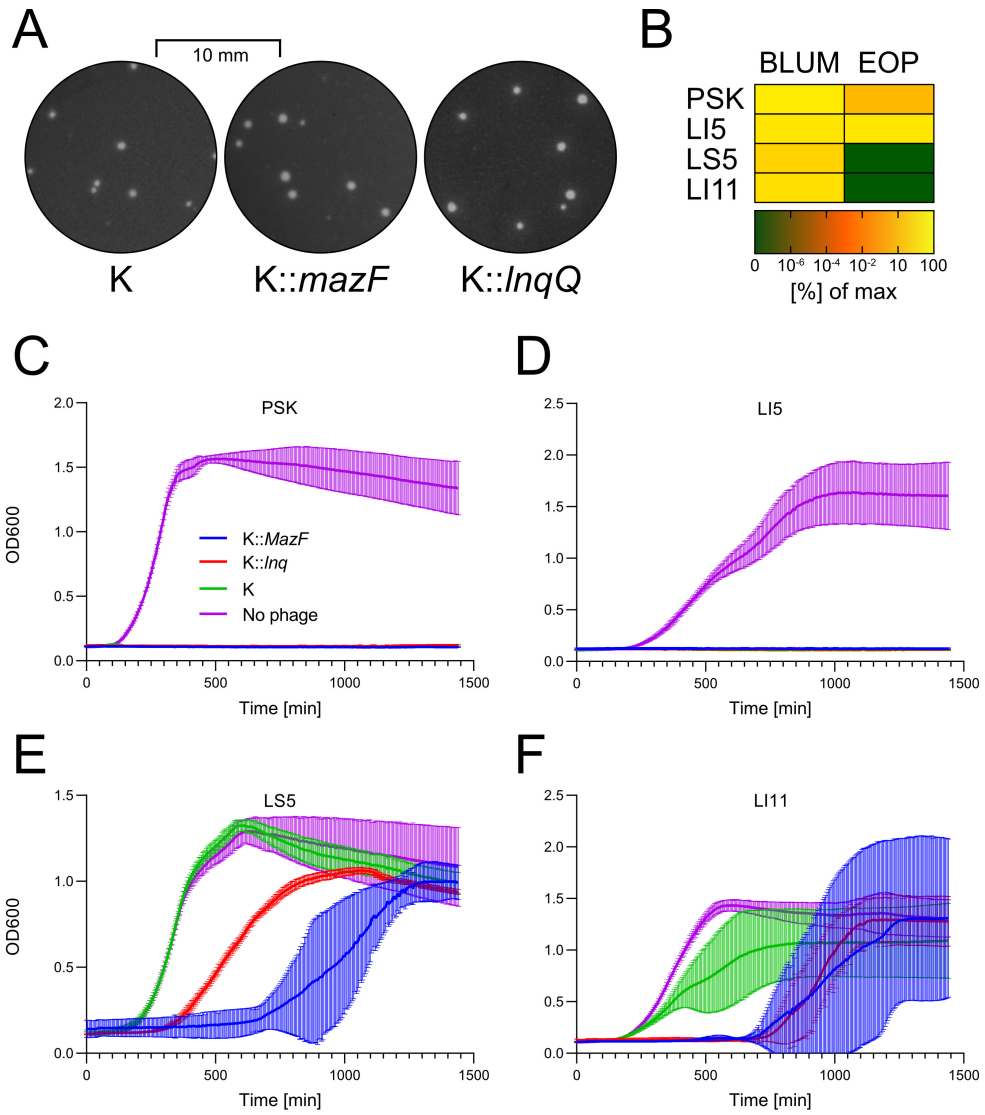


Figure 2: Functional comparison of killing efficacy between the two engineered and wildtype phage.

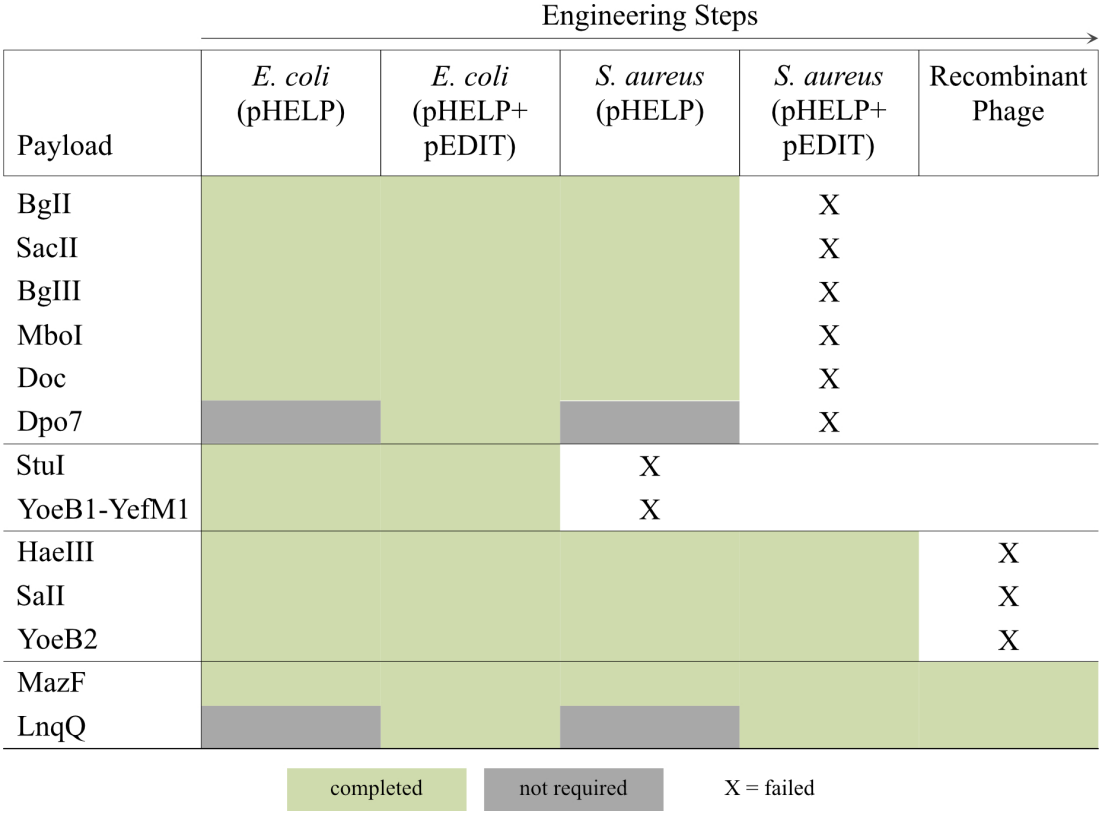
(A) Plaque morphology comparison for the three phages K, K::*LnqQ* and K::*MazF* using soft-agar overlays. **(B)** Bioluminescence (BLUM) and efficiency of plaquing (EOP) for the four selected *S. aureus* isolates determined in a previous study (*see* **Manuscript II**). BLUM for each strain is represented as the mean measured bioluminescence 3h post infection with K::*nluc* (n=3). EOP is given by the mean number of plaque forming units (PFU) after infection with K::*nluc* (n=3). Values are relative to the host with the highest measurement (not shown). **(C-F)** Turbidity reduction assays were performed for the two engineered phages, the wildtype phage and bacterial growth control (no phage) over a course of 24 h at 37°C using 4 different *S. aureus* strains. Bacteria were diluted to OD_{600}=0.005 and infected with $5 * 10^7$ PFU/ml phage. Measurements were performed in technical triplicate for each sample and error bars indicate the standard deviation.

and 12 hours, respectively.

Table 1: Selected payloads, the type of molecule and specific effect on *S. aureus* determined in previous studies.

Payload	Origin	Type	Target
BglII	*Bacillus globigii*	Restriction Enzymes	Species independent, site-specific DNA cleavage
SacII	*Streptomyces achromogenes*		
StuI	*Streptomyces tubercidicus*		
BglIII	*Bacillus globigii*		
MboI	*Moraxella bovis*		
HaeIII	*Haemophilus aegypticus*		
SalI	*Streptomyces albus*		
Doc	*Streptococcus pneumoniae*	Protein Toxins	Translation inhibitor
Yoeb1	*Staphylococcus aureus*		Reduces planktonic growth and biofilm production
Yoeb2	*Staphylococcus aureus*		
MazF	*Staphylococcus aureus*		Sequence-specific mRNA cleavage
LnqQ	*Lactococcus lactis*	Short, leaderless bacteriocin	Membrane perforation
Dpo7	*ϕIPLA7*	Phage pre-neck appendage depolymerase	Biofilm dispersion in *S. aureus*

Table 2: Selected payloads and their status throughout the engineering pipeline.
The first two steps include the transformation of *E. coli* strain XL1-blue MRF' with the immunity plasmid (pHELP) where required, and subsequent transformation with the corresponding homology donor plasmid (pEDIT). The next two steps include the same procedure for *S. aureus* strain RN4220. The final step of obtaining recombinant phage was done by first infecting the homology donor strain RN4220 (pEDIT) or RN4220 (pEDIT+pHELP) and then RN4220 (pSELECT) with the obtained lysate, which was followed by screening for recombinant plaques.



Discussion

The genetic engineering of phages to contain antimicrobial effector payloads targeted against bacterial pathogens has great potential for expanding the possibilities and successful implementations for phage therapy. In this study, we screened a variety of antimicrobials, selected for their previously shown *in vitro* activity against *S. aureus* and other bacteria, for viable introduction into the phage genome and potential enhanced killing efficacy of the engineered phage. We previously engineered a large, lytic *S. aureus* phage, K, to express a bioluminescent reporter payload, which furthermore showed a broad host range against a panel of vancomycin resistant *S. aureus* (VRSA) clinical isolates (*see* **Manuscript II**). The homologous recombination (HR)-driven, CRISPR-Cas9-counterselection (CS)-assisted engineering approach we employed in this previous study proved highly efficient for the simple and rapid construction of recombinant phage. The major capsid protein is typically strongly expressed during phage infection [23–25], and integration of the payload behind this location resulted in high expression levels of the bioluminescent reporter payload (*see* **Manuscript II** and [26]). These factors motivated us to evaluate a variety of antimicrobial effector genes using the same engineering approach. We selected effector genes coding for 14 different compounds with varying mechanisms of action, which have previously been shown to have a strong bactericidal or bacteriostatic effect on *S. aureus* and other bacteria (**Table 1**). Contrary to introduction of the bioluminescent reporter payload, which had no evident negative effect on the integrity of the phage particles or host cell, most of the antimicrobial payloads (where we were able to obtain the engineering strains) did not lead to viable phage progeny, likely due to significant fitness defects for the bacterial host and/or the phage (**Table 2**). The failure to acquire several of the engineering strains is most likely due to residual expression of the payload, which consequently exhibited high toxicity for the host cell. For payloads where we were able to acquire recombinant phages following CS, all but

two did not have correct integration of the intended gene. Our previous engineering efforts and the larger size of the previously used bioluminescent reporter payload (516bp) compared to our payload genes make it unlikely that the payloads were excluded due to size limitation during genome packaging. Likely explanations are either a direct effect of the expressed compound against phage replication or that exceeding toxicity against the host cell led to cell death before assembly of phage progeny could occur. The two engineered phages we were able to obtain, K::*lnqQ* and K::*mazF*, contained genes coding for the short leaderless bacteriocin, lacticin Q [19], and the *S. aureus* protein toxin MazF [22], respectively. We performed turbidity reduction assays with K::*lnqQ* and K::*mazF*, which showed full control of bacterial growth for PSK and LI5. In the case of LS5 and LI11, both K::*lnqQ* and K::*mazF* showed significantly increased killing efficacy compared to wildtype K. Previous experiments using K::*nluc* showed evidence of gene delivery and payload expression, but inability to establish progressive productive infection in several hosts. The significantly elevated levels of growth inhibition of the engineered phages compared to the wildtype in particularly these strains suggests that intracellular expression of LnqQ and MazF after genome delivery leads to cell lysis and phage progeny release, allowing for the infection of neighboring cells to proceed. In future, protein expression levels of the two payloads should be quantified.

Overall, our results show that the arming of *S. aureus* K with antimicrobial effector payloads is non-trivial and that, despite the use of immunity factors during the engineering process, acquisition of the vast majority of engineered phage variants proved unsuccessful using our system. Nevertheless, in those cases where we succeeded, antimicrobial payload integration into K led to significantly increased bactericidal activity over the wildtype phage. Further efforts should be put into the evaluation of K::*lnqQ* and K::*mazF* to further quantify the bactericidal or bacteriostatic effect and the underlying mechanisms, potentially in more advanced infection models.

Materials and Methods

Plasmid and payload design

The *S. aureus* homologous recombination donor plasmid pEDIT was used for the various payloads as previously described in (*see* **Manuscript 2**). Briefly, novel payload genes were inserted behind ribosomal binding site GAGGAGGTAAATATAT flanked by homology arms ranging from 200-400 bp, based on the payload in question. Homology arm sizes were varied to exclude full coding sequences from the phage genome which may result in the production of toxic gene products. We used an *S. pneumoniae*-derived dual-RNA:Cas9-based CRISPR system-coding plasmid (pSELECT) (*see* **Manuscript II** and [27]) as a counterselection system for all engineered phages. Briefly, we exchanged the two sequences of short CRISPR RNA (crRNA) spacers to target 30 bp stretches of DNA with proximal protospacer adjacent motifs (PAM), up- and downstream of each intended insertion site, respectively. Protein sequences of the payloads were obtained from UniprotKB, reverse translated and codon optimized for *S. aureus* while avoiding Rho-independent termination. Optimized genes were ordered as synthetic DNA strings (GeneArt String DNA Fragments (Thermo Fischer) for payload genes, GeneArt Gene Synthesis (Thermo Fischer) for CRISPR spacers) and cloned into the respective plasmids. In those cases where immunity genes mitigating toxic effects of the payload genes were known, these were designed analogously and inserted into an *S. aureus* shuttle vector plasmid pRB474 [28] behind a strong constitutive, *Bacillus subtilis*-derived promoter [29] to obtain the immunity plasmid pHELP.

Bacterial strains and culture conditions

S. aureus strain RN4220 [30] was used as precursor for the engineering hosts RN4220 (pEDIT), RN4220 (pEDIT,pHELP) and RN4220 (pSELECT). *S. aureus* PSK (ATCC 19685) was used for propogation and infection assays with recombinant phage. *E. coli*

XL1-blue MRF' (Stratagene) was used for plasmid amplification prior to RN4220 transformation. RN4220 and XL1-blue cultures were grown over night (O/N) at 37°C in Brain Heart Infusion (BHI) Broth (Biolife Italiana) and Luria-Bertani/Lysogeny broth (LB) medium (3M sodium chloride, 10 g/L tryptone, 5 g/L yeast extract, pH 7.2), respectively.

Soft-agar overlays

BHI bottom agar and LC top agar (LB supplemented with 10 mM CaCl2, 10 mM MgSO4, 10g/L glucose) were used for soft-agar overlays (BHI/LC). Briefly, 5 ml BHI soft-agar were melted, let cool to 47°C, inoculated with 200 μl bacterial culture and 10 μl of phage suspension and spread evenly onto BHI agar plates. Plates incubated at 37°C for 12-18 h.

Bacteriophage propagation

High titer lysates of K were aquired by obtaining plates showing semi-confluent lysis of the bacterial lawn using soft-agar overlay. Plates were supplemented with 5 ml SM buffer (100 mM sodium chloride, 8 mM MgCl2, 50 mM Tris-HCl (pH 7.5)) and left at 4°C for 2 h with constant agitation. The buffer was then removed and centrifuged (10'000 g, 10 min, 4°C) to pellet cell debris. The supernatant was sterilized by filtration using 0.22 μm filters. Open-Top Thinwall Ultra-Clear Tube (Beckman Coulter) were loaded with the following CsCl density layers: 1.7 g/ml CsCl, 1.5 g/ml CsCl, 1.35 g/ml CsCl. The densities were obtained by dissolving CsCl salt in S/M buffer. As the topmost layer, the phage lysate sample adjusted to a density of 1.15 g/ml was added. Ultracentrifugation was performed at 78'200 rcf for 2 h at 10°C. The tubes were then collected and the visible phage band was recovered. The lysate was dialyzed for a minimum of 2x 2 h using the Slide-A-LyzerTM Mini Dialysis Device (Thermo Scientific).

Electroporation

XL1-blue electrocompetent cells were electroporated at 2.5 kV, 200 ω, 25 μF, incubated for 1 h with SOC recovery medium (2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 20 mM Glucose) and plated on selective medium. RN4220 electrocompetent cells were electroporated at 1.8 kV, 600 ω, 10 μF with 500-1000 ng of amplified plasmid DNA, incubated for 1 h in B2 recovery medium and plated on selective medium to isolate successful transformants.

CRISPR-Cas9-assisted engineering of K

For each of the selected payloads, RN4220 was transformed with the corresponding payload homology donor plasmid and CRISPR-counterselection plasmid to obtain the editing and selection strains, respectively. In those cases where immunity plasmids were available, these were introduced prior. To obtain recombinant phage, first the strain containing the homology donor plasmid was infected with K to obtain a high titer lysate. This lysate was subsequently used to infect the counterselection strain. Individual plaques were picked and PCR and sanger sequencing performed to validate correct insertion of the payload. The recombinant phage lysate was passaged on the counterselection strain three further times to ensure monoclonality of the phage population. Lysates were purified prior to efficacy testing.

Turbidity reduction assays

Cultures of all strains used for testing the efficacy of recombinant phage were grown to the stationary phase and subsequently diluted to an OD_{600} of 0.005 into flat-bottom 96-well microtiter plates (Bioswisstech). These were subsequently inoculated with $5 * 10^7$ PFU/ml for each recombinant phage. The turbidity of each well was measured every 5 min at 37°C using a spectrophotometer (SPECTROstar Omega or SPECTROstar Nano, BMG Labtech) over 24 h. Uninfected bacterial dilutions were used as growth controls

and growth medium without bacteria was used as control for background turbidity.

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Chapter 3

Discussion and Outlook

The thesis outlines methods of synthetic biology and genetic engineering in the context of phage therapy, and demonstrates various phage engineering applications directed against the major human pathogen *Staphylococcus aureus*.

Pathogenic bacteria, including *S. aureus*, pose a significant burden for healthcare systems worldwide, which is exacerbated immensely by the continuous emergence of multi-drug resistant strains [29, 33, 34]. The steady decline in antibiotics effective against these pathogens, combined by a lack of financial incentive for pharmaceutical companies to proactively develop new antibiotics, has fueled research into alternative antimicrobials such as phage therapy [30–32, 233–235]. Both naturally occurring phages and genetically engineered variants have successfully been employed in the past for the treatment of bacterial infections [233–235]. There are, nevertheless, a number of caveats associated with phage therapy, especially for the application of natural phages (*see* **section 1.5.2**). Bacteria can rapidly develop resistance to phages, and prolonged treatment with multiple phages is usually necessary to clear an infection. Direct effects of phage-mediated bacterial killing, such as endotoxin release, can also pose a significant danger to the patient.

These types of risks have lead to ethical concerns and numerous regulatory hurdles associated with phage therapy. To move away from individual compassionate use cases towards a paradigm of widespread phage therapy, pharmacodynamic and pharmacokinetic characterization, as well as interactions of phages with the human immune system, are therefore paramount. Further research and clinical testing will hopefully allow for

more effective therapy through better understanding of the precise underlying mechanisms of a particular phage therapeutic. This may also help overcome ethical concerns and regulatory challenges associated with phage therapy.

For individual, compassionate use cases, much effort is already put into identifying phages suitable for clinical application. The emergence of phage banks and high-throughput isolation and characterization techniques have great potential in aiding this endeavour [469]. This also has positive implications for the growing number of phage banks, which are globally dispersed, have only a relatively low number of phages, with little to no standardization and collaboration between the individual institutions and thus far have only contributed to a small number of compassionate use cases [470]. The expansion of these phage banks, combined with thorough characterization of the individual phages, will greatly enhance the ability to identify individual phages suitable for specific applications or rationally design phage cocktails tailored to cover a wide range of bacterial pathogens and resulting infections. A future is imaginable where a bacterial sample isolated from an antibiotic resistant infection is screened against a large panel of phages provided by such phage banks, such that matching phages can be rapidly identified and deployed for clinical use.

Engineered phages are another approach to overcome a number of caveats associated with natural phage therapy. This includes, but is not limited to, the expansion of host range, mitigation of bacterial resistance emergence and delivery of antimicrobial effector payloads for enhanced bactericidal activity (*see* **section 1.6**). Engineered phages are an entirely new field of research, and clinical applications have thus far been largely limited to methods such as lytic conversion [235]. Engineered phages also have numerous limitations and only very few clinical trials employing engineered phages have been conducted to date [384, 471]. Specifically the introduction of heterologous effector payloads into phage scaffolds to enhance bactericidal efficacy will likely complicate the regulatory frameworks associated with phage therapy even further. There are also ethical concerns

associated with the use of recombinant phages for therapy, especially concerning the uncontrolled release of genetically modified phage variants into the environment [472, 473]. Furthermore, despite there being no evidence that bacteriophages can infect eukaryotic cells directly, their have been reports of interactions with eukaryotic cells, which must be considered before widespread administration of phage therapy becomes the norm [474, 475]. Engineering of phages can prove difficult, and different approaches are necessary based on the phage/host pair in question, as well as the genetic alteration intended to be introduced into the phage. Consequently, there is a pressing need for novel, diverse methodologies to expand the toolbox for phage engineering.

Homologous recombination (HR) and synthetic biology are the the two main methods used in phage engineering. In **Manuscript I**, we review various different engineering methodologies as well as potential applications and future outlooks therefor. As mentioned before, the method of choice has to be selected and potentially adapted based on the phage and/or payload in question. In this thesis, we employed both methods to produce recombinant phages, with HR-directed, CRISPR-Cas9-assisted counterselection (CS) used in **Manuscripts II, IV and V**, as well as synthetic genome assembly and reactivation in L-form bacteria **Manuscript III**.

Synthetic methods are based on the generation of synthetic gene fragments, followed by genome assembly and reactivation in a suitable host bacterium. This method has the great advantage of producing purely recombinant phage with no necessity for a downstream selection step. E.g., in Gram-negative bacteria, reactivation can be easily achieved via transformation of the host bacterium with assembled phage DNA. This proves more difficult for many Gram-positive species, such as *S. aureus*, where transformation efficiencies are exceedingly low barring a small number specifically tailored laboratory strains, typically cured of prophages and intracellular restriction mechanisms. In **Manuscript III**, we demonstrate the conversion of Gram-positive bacteria to cell-wall deficient L-form variants, which can be used to reboot synthetic phage genomes. This was achieved for

two different *Staphylococcal* species, *S. aureus* and *S. xylosus*, as well as *L. monocytogenes*. This method has even been shown to be able to reboot phages from a different genus than the host bacterium (**Manuscript III** and [366]). Several caveats exist for synthetic engineering approaches. For one, genome assembly can be difficult to achieve. Mycobacteria, for example, have high G/C content of around 61% - 71% which can hamper fragment synthesis due to difficulties in primer design and the formation of secondary hairpin structures in the DNA [476]. Furthermore, even state-of-the-art genome assembly techniques such as TAR-cloning and Gibson isothermal assembly have difficulties in achieving the error-less assembly of large genomes [477, 478]. As we demonstrated in **Manuscript III**, not every L-form can reboot phage DNA, and not every phage can be rebooted in a given L-form. The factors governing "rebootability" are widely unknown and difficult to elucidate. This requires arduous screening of cross-compatible phage genomes and L-forms, especially when considering this technology to be used in future high throughput phage engineering pipelines. Future developments such as cell-free transcription-translation (TXTL) systems have great potential, as they can be better standardized than L-forms, and components can be altered and combined to expand the spectrum of rebootable phage genomes [479]. Novel assembly and DNA delivery methods are constantly being revised and further developed and will hopefully significantly contribute to future phage engineering endeavours.

HR consists of the delivery of an homology donor template, typically encoded on a plasmid, into a suitable host cell, which is subsequently infected with the to-be-engineered phage. If HR is successful, a small fraction of the phage progeny will contain the intended alteration. This necessitates a subsequent selection step in order to isolate recombinant from wildtype phages. This is arduous, especially in strains with low recombination frequency, where methods such as CRISPR-Cas9 CS or the screening for bioluminescent plaques are necessary. For this reason, this method is typically only employed when synthetic approaches are not viable. We demonstrate the successful implementation of an

HR-directed and CRISPR-Cas9 CS-assisted pipeline for the engineering of a broad host range, *S. aureus*-infecting *Kayvirus*, phage K (*see* **Manuscript II** and **Manuscript V**). Although our CS system was highly efficient in this case, **Manuscript IV** demonstrates that this is wildly dependent on the location of the genetic payload and/or CRISPR spacer design and rationale selection of insertion sites and design of CRISPR spacers is essential for success.

The introduction of reporter-protein coding genes and antimicrobial effector payloads allows construction of phages with tunable function for various applications. Provided a suitable engineering system is available for the intended phage/host combination, phages can be engineered in a variety of ways which have been successfully demonstrated in the past. One such example are reporter phages, which are engineered to emit a signal upon infection of a bacterial host and have been developed for a variety of strains in the past. Reporter phages have numerous potential applications including food quality control and clinical diagnostics [394]. There are several limitations for this approach, including the fact that detection of bacterial pathogens is limited to the host range of the engineered phage. Furthermore, there can be complex matrix effects in food and other products, such as milk, which can significantly reduce the ability of phages to infect bacteria [480]. In **Manuscript II** we developed an *S. aureus*-infecting reporter phage and outline how some of these limitations can be overcome. By using phage K as an engineering backbone, we achieved an exceedingly high detection range, successfully detecting the entire range of 71 *S. aureus* strains included in our study. Nevertheless, this extensive host range can also have negative implications, such as the false positive detection of other *Staphylococcus* species such as *S. epidermidis*, which has also been shown to be susceptible to K [481]. We were able to overcome the previously mentioned negative matrix effects in raw bovine milk by optimizing the assay through dilutions of infected milk samples in a manner to still allow for phage infection while maintaining detectable bacterial levels. The application of reporter phages in clinical diagnostics such

as *S. aureus*-induced bacteremia are limited, as in these cases patient treatment has to proceed immediately without prior species-typing. Nevertheless, reporter phages show great promise as e.g., a companion diagnostic tool for phage therapy [482].

A further approach with wide implications for the future of phage therapy is the engineering of phage to contain antimicrobial effector payloads which are expressed within the host bacterium following phage infection. This method has been successfully employed in applications such as catheter induced urinary tract infections [355], and several other programs employing a variety of antimicrobial effectors, some of which are currently undergoing clinical evaluation [378, 379, 483, 484]. The incorporation of antimicrobial effector payloads into the phage genome is typically more difficult than e.g., reporter payloads (such as bioluminescent protein-coding genes) which have no direct effect on the host cell metabolism. Conversely, antimicrobial payloads can have a significant impact on the phage and/or host and rational payload selection is paramount. Multiple factors which can lead to failure to obtain recombinant phage have to be considered. Depending on the size of the phage and payload, packaging defects can occur if the resulting recombinant phage genome is significantly larger than the wildtype variant. Payloads that require post-translational modifications may not be active, depending on the compatibility with the producing host organism. Toxic payloads may affect the viability of the host and/or phage and therefore severely affect phage replication. Such growth defects can prevent plaque formation (and therefore phage isolation) and curb yields during phage production. A simple strategy to deal with payload toxicity is the use of an "immunity" protein that mitigates the toxic effect of a given payload. To this end, a suitable immunity gene must be delivered into the host cell in addition to the homology donor template. Although these immunity factors are intended to protect the cell from the effects of residual expression of the toxic payload gene encoded on the donor plasmid, the expression of these immunity factors may also have toxic effects for the cell. Plasmid compatibility between the the immunity plasmid and the homology donor plasmid

also has to be taken into account. There is a complex interplay between phage infection and replication, payload expression and host cell metabolism. Toxic payloads which are expressed at very high levels early in the infection cycle may lead to cell death before production and release of phage progeny can occur. In **Manuscript V**, we screened a large number of payloads, only two of which resulted in viable recombinant phages. Failure to engineer phages containing the remaining payloads can likely be attributed to factors described above. Nevertheless, the two recombinant phages we were able to obtain showed elevated bactericidal activity when compared to the wildtype phage. We demonstrate that, by rationale selection and functional screening of numerous payloads, this approach is viable even for difficult to engineer phage/host combinations such as *S. aureus* and phage K.

Although the arming of phages with antimicrobial payloads can be arduous, as outlined here, our results still have strong implications for the potential future of phage therapy. For one, high throughput engineering pipelines could exhaustively screen payloads for viability and efficacy. Revolutionary computational methods such as Alphafold 2 [485], as well as associated domain-domain interaction predictions and corresponding metabolic network inference could lead to sophisticated modelling of the effect a payload will have on host cell metabolism. In **Manuscript IV**, we developed a computational approach to aid in phage engineering by employing machine learning (ML) to develop a computational pipeline in order to elucidate promoters and associated transcription strength scores from genetic sequence data alone. Not only did our computational predictions match previously experimentally determined promoters, but payload expression, benchmarked via integration of the nanoluciferase gene, corresponded with our predicted expression levels.

In general, these types of computational tools can greatly assist in enhancing existing phage engineering methodologies and will likely dictate the direction of phage engineering and therapy in the next decades. Next to computationally aiding in determining

rationale design guidelines, the mining of phage banks for host characteristics, especially sequence data, has several implications for the future. Phage-host matching can lead to patient tailored treatments, as is already done experimentally. Computational and high-throughput screening pipelines could vastly accelerate the identification and distribution of suitable phages for individualized patient treatment. The machine-learning powered elucidation of genetic components deterministic of host specificity is almost inevitable, given the vast influx of genomic sequence data, and in the future will likely aid significantly in predicting phage-host pairs as well as allowing for engineering applications such as host range reprogramming. Finally, the "holy grail" of phage therapy is on the horizon, and deeper insight into phage/bacterium genomic landscapes will potentially allow for *in silico* prediction and *de novo* synthesis of novel, fully synthetic phage variants.

With this work, we have outlined, analyzed and established a multitude of phage engineering applications directed against the major human pathogen *S. aureus*. Through the development of novel engineering pipelines and engineered phages with the possibility for the future application in phage therapy, I hope to have significantly contributed to the field of phage research, engineering and therapy. It is with cautious optimism that we can look into a future where the "golden age of antibiotics" is superseded or complemented by a similar "golden age of phage therapy".

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Acknowledgements

First, I would like to thank Martin for giving me the possibility to conduct my PhD studies in his group and thereby delve into this exciting field of research. My great thanks goes out to Sam for his excellent supervision, teaching and general guidance throughout my PhD. Thanks also go to Dr. Alexander Harms for co-refereeing my thesis. My deepest gratitude goes to all the students who I had the great pleasure of supervising, each of which conducted excellent work and was a joy to be around: Cheickna, Moritz, Jasmin, Sarah, Jeannine, Isabel, Julien and Emmy—couldn't have done it without you. Thank you also to my collaborators at the Balgrist hospital, Shawna and Lorenz, for their contributions and making one of our exciting projects possible. Lot's of love goes toward past and current lab members. Pauline, Anja and Léa for our countless science and non-science (and non-sense) conversations, as well as constant moral support. Thanks to Eric for helping me scheme my marriage proposal and both Eric and Christian G for granting me an outlet for the more nerdy (especially space related) topics. Thanks to Anna and Christian for always making me feel at home, especially during my early times in the group, and Matt for always throwing in his two pence when needed. Special thanks also to Carmen, Christian G and Rasha for always being there to help in the lab and simply being lovely people in general. SpeciaL thanks to the *Royal Society of L-forms* and all it's members, especially Alex, Marco and Sämi for all the laughs. Special thanks also to Uschi the cow for her invaluable scientific contribution. My greatest thanks is reserved for my family: Mom, Dad, Annika, Meike and Daria, your unwavering moral support has always granted me guidance in the darkest times and love and laughter in the lightest. And finally, to the love of my life, Sonja, who has stuck with me through thick and thin and always gives me the feeling that I can shoot for the stars.

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